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Calbrain, a Novel Two EF-hand Calcium-binding Protein That Suppresses Ca^{2+}/Calmodulin-dependent Protein Kinase II Activity in the Brain*

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A cDNA clone that encodes a novel Ca^{2+}-binding protein was isolated from a human brain cDNA library. The gene for this clone, termed calbrain, encodes a 70-amino acid polypeptide with a predicted molecular mass of 8.06 kDa. The analysis of deduced amino acid sequence revealed that calbrain contains two putative EF-hand motifs that show significantly high homology to those of the calmodulin (CaM) family rather than two EF-hand protein families. By Northern hybridization analysis, an approximate 1.5-kilobase pair transcript of calbrain was detected exclusively in the brain, and *in situ* hybridization study revealed its abundant expression in the hippocampus, habenular area in the epithalamus, and in the cerebellum. A recombinant calbrain protein showed a Ca^{2+} binding capacity, suggesting the functional potency as a regulator of Ca^{2+}-mediated cellular processes. Ca^{2+}/calmodulin-dependent kinase II, the most abundant protein kinase in the hippocampus and strongly implicated in the basic neuronal functions, was used to evaluate the physiological roles of calbrain. Studies *in vitro* revealed that calbrain competitively inhibited CaM binding to Ca^{2+}/calmodulin-dependent kinase II (K_i = 129 nM) and reduced its kinase activity and autophosphorylation.

Calcium ion (Ca^{2+}) is a universally employed cytosolic messenger in eukaryotic cells. It is involved in many cellular processes such as signal transduction, contraction, secretion, and cell proliferation (1, 2). In the central nervous system, Ca^{2+} plays a major role in the activities and functions of neuronal cells (3–5). One of the most widely recognized roles of Ca^{2+} in synaptic function is its action in neurotransmission. Studies on the effects of Ca^{2+} on neurotransmitter release, synaptic protein phosphorylation, synaptic vesicles, and synaptic membrane interactions have provided experimental evidence that Ca^{2+} regulates several biochemical and morphological events in synaptic preparations (6, 7).

In many cases, the effects of Ca^{2+} are mediated by the Ca^{2+}-binding proteins (8). One superfamily of these proteins is the EF-hand protein family. The EF-hand proteins are characterized by single or multiple pairs of a common helix-loop-helix motif that coordinates Ca^{2+} (9, 10). For instance, CaM,[1] troponin C, and myosin light chain have four EF-hand motifs/molecule, whereas S100 proteins have only two motifs per molecule. In addition to the role as a Ca^{2+}-buffering system, the binding of Ca^{2+} causes a conformational change of EF-hand proteins and enables them to interact with their target proteins. Most of the EF-hand proteins except CaM show specific tissue distribution, suggesting their particular functions in each tissue. CaM has broad distribution within the cell and throughout different tissues and is a multifunctional regulatory protein that, in a Ca^{2+}-dependent manner, activates a number of enzymes that are involved in a variety of physiological processes (11). Among these enzymes, CaM-kinase II is one of the most abundant Ca^{2+}-activated protein kinases in the brain, and it plays important roles in a variety of neural functions including receptor function, neurotransmitter release, and synaptic plasticity (12).

CaM-kinase II is activated by binding to the Ca^{2+}-bound form of CaM, which dramatically increases the affinity of the enzyme for Mg^{2+}/ATP, thus leading to substrate phosphorylation and autophosphorylation (13–15). This self-regulation system coupled to Ca^{2+}/CaM-dependent autophosphorylation may be involved in important physiological roles responding to transient elevation of intracellular Ca^{2+} (16). During autophosphorylation (of Thr^{286}), trapping of CaM in CaM-kinase II occurs, resulting in prolongation of the activation period of CaM-kinase II. This process is understood as a good model of memory formation (15, 17).

In the present study, we cloned and characterized a novel two EF-hand Ca^{2+}-binding protein, termed calbrain, that is brain-specific and highly expressed in the hippocampus. To characterize the physiological function of this protein in the hippocampus, the effects of calbrain on CaM-kinase II were examined. The results revealed that calbrain competitively inhibited the activity of CaM-kinase II, suggesting that calbrain may be involved in neuronal signal transduction and memory.

EXPERIMENTAL PROCEDURES

Polymerase Chain Reaction and Human cDNA Library Screening—Degenerate primers were originally designed from transmembrane regions of several mammalian G-protein-coupled receptors. Polymerase chain reaction was performed with these primers using 100 ng of human brain cDNA library (CLONTECH) as a template. The cycling condition was 1 min at 95 °C, 1 min at 55 °C, and 0.5 min at 72 °C for 37 cycles. The polymerase chain reaction products were end-repaired

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) X94700.

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[1] The abbreviations used are: CaM, calmodulin; CaM-kinase, Ca^{2+}/calmodulin-dependent protein kinase; PAGE, polyacrylamide gel electrophoresis; MOPS, 4-morpholinepropanesulfonic acid.

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1    GGCGCTGCAGGTTAGAGCCTGGCAATGCCGTTTGGGTGTGTGACTCTGGGCGACAAGAAG   60

61   AACTATAACCAGCCATCGGAGGTGACTGACAGATATGATTGGTGTAAAGGAACTGCGAGA  120
                                        M  I  G  V  K  E  L  R  D
                                   EF-1
121  TGCTTTCCGAGAGTTTGACACCAATGGTGATGGGGAAATAAGCACCAGTGAGCTGCGAGA  180
      A  F  R  E  F  D  T  N  G  D  G  E  I  S  T  S  E  L  R  E

181  GGCTATGAGGAAGCTCCTGGGTCATCAGGTGGGACACCGAGACATAGAGGAAATTATCCG  240
      A  M  R  K  L  L  G  H  Q  V  G  H  R  D  I  E  E  I  I  R
                                   EF-2
241  AGATGTGGACCTCAATGGGGATGGACGAGTGGACTTTGAAGAGTTTGTCCGGATGATGTC  300
      D  V  D  L  N  G  D  G  R  V  D  F  E  E  F  V  R  M  M  S

301  CCGCTGAGGCCGCGAGGGCCCCTCCAGGACTGCCAAGCTCCCAAAGGCGGGGCTAAGAGG  360
      R  *

361  AGCTAGAGCTTGCCTCACCCGCTGTATCCGCCGAGAGCCCAGGATGTACTGGCGGATGGG  420

421  GCCTGCCTGCACCCCGGGGAGGCGCCCACCCGGGACCCCACCCCTCCGCACTGTGAAAGA  480

481  CTAACTCCTGCAACTGGAAAGCGGGGGCGCCCGCCGACGAGGAGGCCACCGTGCCAAGCC  540

541  GGCAGAGGTCATGCCAGGCGCCAAGGGCAATGTGCCCAGCTGCTGCTGGCTGGGTGGGCC  600

601  AGGGAGCCCGCCAGCAGACCCCACACAGCATGTCCGCCCCAGGGCAAAGCTTCCCACTTT  660

661  CGTT  664
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FIG. 1. **Nucleotide sequence and deduced amino acid sequence of human calbrain.** The sequence was derived from human brain cDNA clones. Two EF-hand domains are indicated by *lines*; the in-frame methionine and stop codon are shown in *bold face type*.

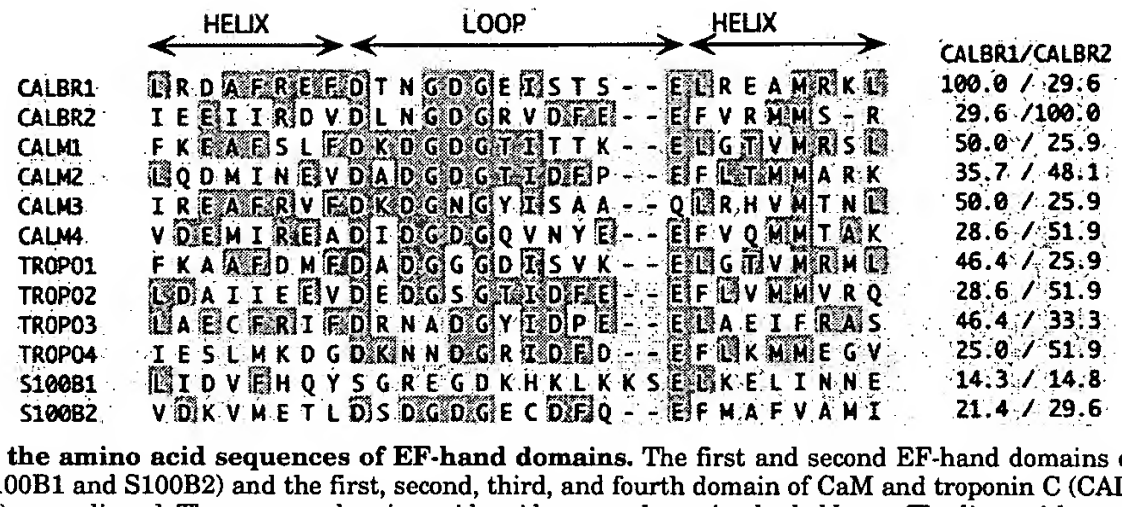

FIG. 2. **Alignment of the amino acid sequences of EF-hand domains.** The first and second EF-hand domains of calbrain (CALBR1 and CALBR2) and S100β (S100B1 and S100B2) and the first, second, third, and fourth domain of CaM and troponin C (CALM1 to CALM4, TROPO1 to TROPO4, respectively) were aligned. The conserved amino acid residues are shown in *shaded boxes*. The *lines* with *arrows* indicate the helix and loop region of EF-hand. The *numbers* on the right indicate the percentages of similarity between either CALBR1 or CALBR2 and EF-domains in each protein.

with T4 DNA polymerase and ligated into pCR-Script SK(+) vector (Stratagene). Samples were transformed, and colonies were picked for subsequent sequencing. The sequence data was obtained using ABI 373 sequencer (Perkin-Elmer) with dye-terminators by the method of Sanger *et al.* (18). The data was searched against SWISSPROT (Ver.30.0) data base by BLAST algorithm (18). The fragment that showed a similarity to Ca^{2+}-binding proteins was chosen for further analysis.

About one million recombinants of human brain λgt11 cDNA library (CLONTECH) were grown and transferred onto nylon membranes. The polymerase chain reaction fragment was labeled with [α-^{32}P]dCTP and used as a probe for the screening. Hybridization-positive plaques were picked and grown to purify the DNA. Inserts from those positive clones were subcloned into *Eco*RI cloning site of pBluescript KS (+) vector (Stratagene). The sequence was determined, and data was searched as above.

Northern Hybridization Analysis—The full-length coding region of calbrain was labeled with [α-^{32}P]dCTP and used as a probe for Northern hybridization analysis. A human multiple tissue Northern blot filter (CLONTECH) containing 2 μg of poly(A)+RNA in each lane was pre-hybridized in a solution containing 5× saline/sodium phosphate/EDTA, 10× Denhardt's, 100 μg/ml salmon sperm DNA, 50% formamide, and 2% SDS for 3 h at 42 °C. The blot was then hybridized in the same solution with the labeled probe at 42 °C for 20 h. The blot was washed several times with a mixture of 2× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate) and 0.05% SDS for 30 min at room temperature and then once with a mixture of 0.1× SSC and 0.1% SDS for 40 min at 50 °C. The filter was exposed to Fuji BASIII imaging plates (Fuji Biomedical, Japan), and the image was analyzed by the BAS 1000 phosphor-imaging system (Fuji Biomedical, Japan).

In Situ Hybridization Study—Adult Sprague-Dawley rats (all male, 8–9 weeks old) were anesthetized, and brains were perfused with phosphate-buffered saline. Brains were then quickly removed and frozen at-80 °C. Cryostat sections (10 μm-thickness) were mounted onto slides. The sections were fixed in 4% paraformaldehyde in phosphate-buffered saline, incubated with 0.01% proteinase K in Tris-HCl for 30 min, and then acetylated for 15 min with 0.2 M triethanolamine containing 0.2% acetic acid. After sequential dehydration through a graded alcohol series, each section was hybridized either with a digoxigenin-labeled antisense RNA probe or with a labeled sense probe as a control. The RNA probe was made from a rat homologue of calbrain gene that is 99.0% identical in nucleotide level to the human one. After hybridization at 50 °C overnight, the slides were incubated with RNase A (10 μg/ml) and washed with 2× SSC and then with 0.2× SSC. Slides were incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim) for 30 min, washed, and developed by incubating at 4 °C overnight with 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium.

Expression and Purification of Recombinant Protein—Recombinant calbrain protein was expressed using the QIA expressionist system (QIAGEN). The gene construct encoding calbrain was subcloned into pQE expression plasmid vector and transformed into *Escherichia coli* host strain M15. Transformed M15 cells were cultured in 500 ml of LB medium containing 100 μg/ml ampicillin and 25 μg/ml kanamycin until the A_{600} of 0.7 at 37 °C. After adding isopropyl-1-thio-β-D-galactropyanoside to a final concentration of 1 mM, the cells were incubated for another 5 h. As a negative control, a plasmid vector with no insert was transformed and expressed in the same way. The cells were harvested and lysed with 10 ml of lysis buffer containing 1 mg/ml lysozyme before sonication. After removal of cellular debris, the supernatant was collected, and histidine-tagged calbrain protein was purified on nickel nitrilotriacetic acid resin in a batch procedure. The protein-resin com-

plex was set into a column and washed with washing buffer containing 50 mM sodium phosphate, 300 mM NaCl, 10% glycerol, pH 5.7. After washing, the protein was eluted with 0.1–0.5 M imidazole gradient in washing buffer.

Tricine-SDS-PAGE and Electrophoretic Transfer—Tricine-SDS-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) was carried out as described previously (19). Protein samples, CaM protein as a control, and molecular weight markers were denatured and applied to 10% Tricine-SDS-PAGE. Separated protein bands were either stained with Coomassie Brilliant Blue or transferred to a nitrocellulose membrane by the method of Kyhse-Andersen (20).

Detection of Calcium Binding by $^{45}Ca^{2+}$—The calcium binding study using $^{45}Ca^{2+}$ was performed according to the method of Maruyama *et al.* (21). Briefly, after the protein transfer, the membrane was soaked in a solution containing 60 mM KCl, 5 mM $MgCl_2$, and 10 mM imidazole-HCl (pH 6.8). The membrane was then incubated in the same buffer containing 1 mCi/liter $^{45}CaCl_2$ for 10 min. Nonspecifically bound $^{45}Ca^{2+}$ was removed by washing with 50% ethanol for 5 min, and dried membrane was exposed to imaging plates. The images were analyzed by Fuji phosphor-imaging system as above, under the Northern hybridization analysis.

Ca^{2+} binding affinity of recombinant calbrain was determined by equilibrium dialysis. Calbrain was first dialyzed overnight against 1000 volumes of a solution containing 150 mM KCl, 10 mM MOPS, pH 7.1, 3 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1 mM EGTA to remove bound Ca^{2+} from the protein. The dialyzed protein was then used for equilibrium dialysis as follows. A 0.5-ml portion of protein at the concentration of 1 mg/ml was dialyzed with shaking for 48 h at 4 °C against 100 ml of the same solution as described above for Ca^{2+} binding affinity but containing various amounts of $CaCl_2$ and $^{45}Ca^{2+}$ (5 μCi) to achieve the desired free-Ca^{2+} concentration. The solutions outside and inside the dialysis tubing were removed, the absorbance at 278 nm was determined, and the protein concentration calculated ($A^{1\%}_{278}$ = 0.958). Samples of these solutions were subjected to liquid scintillation spectrometry. The association constants for metal and H^+ binding to EGTA were based on values measured by Fabiato (23).

Kinase Activity and Autophosphorylation Assay—The activity of CaM-kinase II was assayed by measuring the Ca^{2+}-dependent phosphorylation (^{32}P incorporation) of syntide-2 substrate as described previously by Ochiishi *et al.* (24). The standard reaction mixture contained 50 μM[γ-^{32}P]ATP, 8 mM $Mg(CH_3COO)_2$, 20 μM syntide-2, 0.25 mM $CaCl_2$, 0.1 mM EGTA, 50 mM HEPES buffer, pH 8.0, and 0.3 μg/ml CaM-kinase II (purified from bovine brain, kindly provided by Dr. Yamauchi (25)). A range of CaM or recombinant calbrain (0–200 nM) was employed in the assay mixture at the total volume of 20 μl. The reaction was carried out at 30 °C for 1 min and stopped by spotting onto P81 filter paper. The filter papers were washed several times with 75 mM phosphoric acid, and radioactivity was measured by a liquid scintillation counter. An inhibition assay of Ca^{2+}-dependent activity of CaM-kinase II was performed in the standard mixture as described above in the presence of

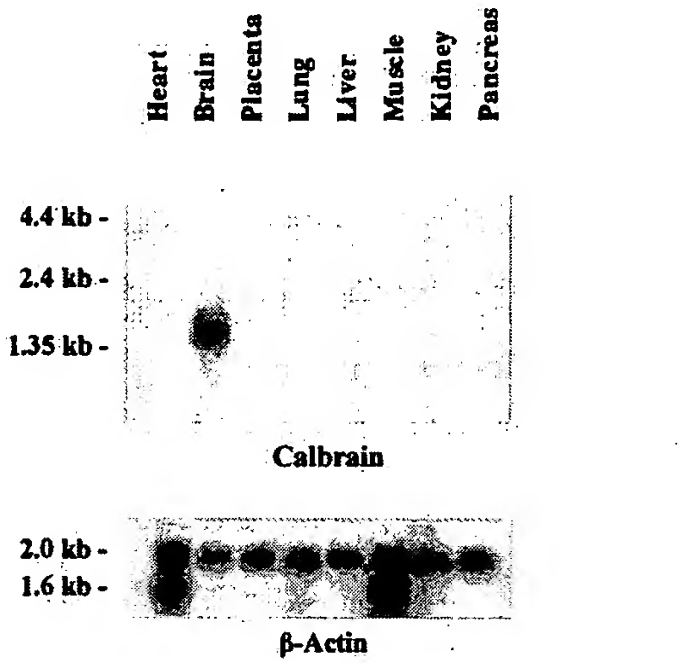

FIG. 3. **Northern blot analysis of the tissue expression pattern of calbrain.** A Northern blot filter containing 2 μg of human poly(A)$^+$ RNA (CLONTECH) was hybridized with ^{32}P-labeled calbrain probe and washed following the protocol of Church-Gilbert. *Numbers* on the left refer to the size of RNA standard run in parallel. ^{32}P-Labeled β-actin was hybridized on the same Northern blot filter as a quantitative control. A single transcript was visualized in the brain sample *lane* (approximately 1.5 kilobases). No signal was detected in other tissues examined. *kb*, kilobases.

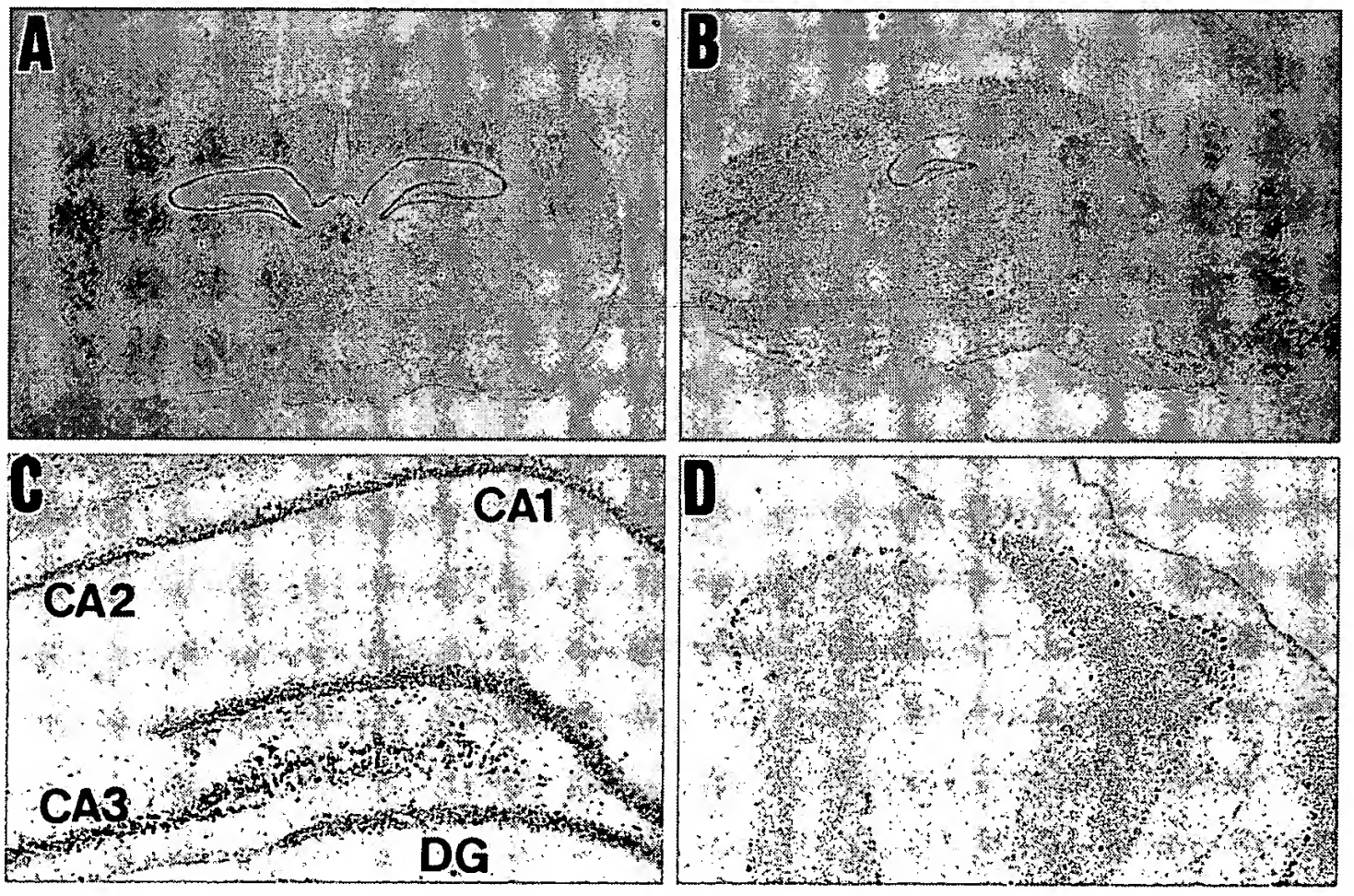

FIG. 4. **Localization of calbrain mRNA in rat brain.** *In situ* hybridization was performed. A rat homologue of calbrain probe (99.0% identical to human one) was labeled and hybridized to a coronal section (*A*) or a sagittal section (*B*) of adult rat brain. The hippocampal area (*C*) and the cerebellar cortex (*D*) are shown in higher magnification. The strong signals were detected in the CA1 to CA3 of the hippocampal gyrus and granular layer of the dentate gyrus (*DG*) in the hippocampus (*C*). In the area of cerebellum, the Purkinje cell layer was stained intensively (*D*).

various concentrations of CaM. Autophosphorylation of CaM-kinase II was assayed in the standard mixture at the total volume of 60 μl. The reactions were carried out at 0 °C for 10 min as described previously (26), then samples were boiled for 3 min and were subjected to SDS-PAGE. After electrophoresis, the gel was stained with Coomassie Brilliant Blue, and the band of CaM-kinase II protein was excised from the gel. Radioactivity of the gel was determined by a liquid scintillation counter.

Inhibition of CaM Binding to CaM-kinase II by Calbrain—The effect of calbrain on CaM binding to CaM-kinase II was examined as described previously (27) with some modifications. The reaction mixture for the binding studies was the same as that used in the kinase activity assay without ATP. Various concentrations (0–200 μM) of ^{125}I-CaM and calbrain were incubated in the reaction mixture at the total volume of 200 μl at 4 °C for 1 h, and they were reacted with 3 μg of polyclonal anti-CaM-kinase II antibody (Transduction Laboratories) at 4 °C for 16 h. The amount of antibody was proved to be adequate for binding to entire CaM-kinase II in the reaction mixture. Then, 10 μl of protein G-Sepharose (Amersham Pharmacia Biotech) was added to each reaction mixture and incubated at 4 °C for 1 h on a rotating wheel. After 3 washes with the reaction mixture without CaM-kinase II, these samples were centrifuged (5000 rpm, 5 min), and the radioactivity of each pellet was measured by a gamma counter. Double-reciprocal plots and determination of K_i values were performed as described by Segel (28).

RESULTS

Calbrain Has Two EF-hand Motifs—The isolated clone was found to have a 210-nucleotide open reading frame that encodes a 70-amino acid polypeptide with a predicted molecular mass of 8.06 kDa (Fig. 1). We named the clone as calbrain. A motif search suggested the presence of two EF-hands, a motif known to be involved in calcium binding (29). By a data base search, calbrain showed significant high homology to CaM and troponin C proteins. The four EF-hand domains of CaM, troponin C, and two EF-hand domains of S100β protein and calbrain were aligned, and the amino acid sequences were compared as shown in Fig. 2. The first domain of calbrain is highly (50.0% each) homologous to the first and the third domain of CaM. The second domain of calbrain is homologous to the second and the fourth domains of CaM (48.1 and 51.9%, respectively) and those of troponin C (51.9% each). The first and the second domains of calbrain showed only 29.6% homology. Although calbrain has only two EF-hand motifs, the similarity between calbrain and two EF-hand protein (S100β) is very low (Fig. 2).

Brain-specific Expression of Calbrain mRNA—From the results of Northern hybridization analysis under high stringency conditions, an approximate 1.5-kilobase single calbrain transcript was detected exclusively in the brain (Fig. 3). No band was detected in other tissues examined including heart, placenta, lung, liver, muscle, kidney, and pancreas.

Localization of Calbrain mRNA in the Brain—Results of *in situ* hybridization study for localization of calbrain mRNA in rat brain was conducted on rat brain sections showed strong signals in the pyramidal layers CA1 to CA3 of the hippocampal gyrus and the granular layer of the dentate gyrus in the hippocampus (Fig. 4). The habenular nucleus in the epithalamus was also stained strongly. In the cerebellum, the Purkinje cells were strongly stained. There were also weak and scattering signals in the cerebral cortex and other areas of the brain. No staining was observed when a sense probe was used as a control.

Ca^{2+} Binding Property of Calbrain—The purified calbrain and CaM as a control were run on a 10% Tricine-SDS-PAGE gel with molecular mass markers (Fig. 5*A*). The molecular mass based on the amino acid sequence of mammalian CaM is 16.7 kDa, which is approximately double the size of calbrain. On Tricine-SDS-PAGE gel, calbrain protein was observed at approximately 8 kDa, and CaM is observed as the same size of its molecular mass. The control fraction (sample using the nonrecombinant vector) on the same Tricine-SDS-PAGE gel did not show any protein bands. The $^{45}Ca^{2+}$ binding study of calbrain are shown in Fig. 5*A*. Both CaM and calbrain showed strong radioactive bands at the expected positions, indicating the binding of $^{45}Ca^{2+}$ to these proteins. Fig. 5*B* shows a saturation curve for the Ca^{2+} binding to calbrain. Scatchard analysis (30) of the data (Fig. 5*B*, *inset* and legend) reveals that, in the

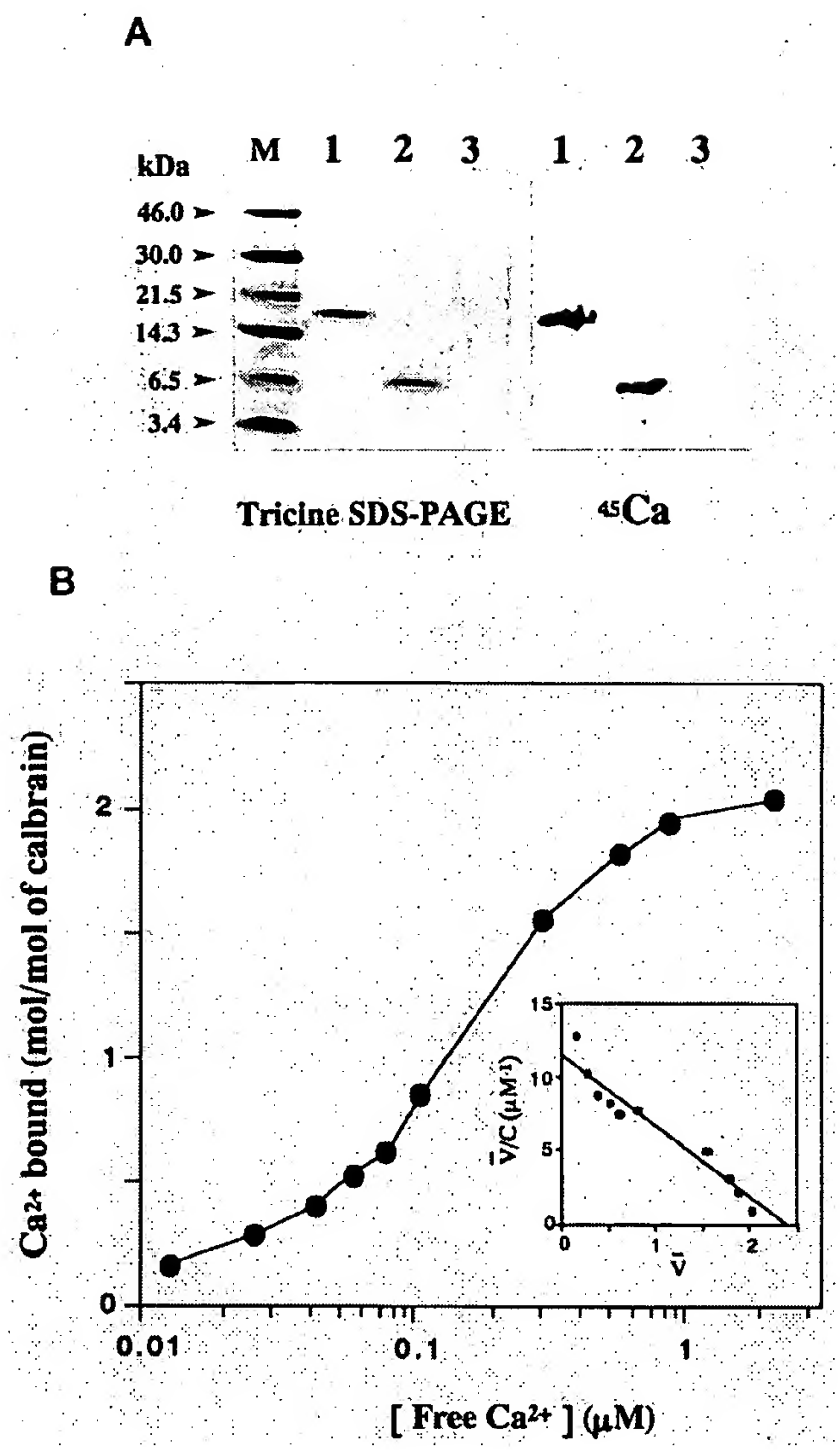

FIG. 5. **Purification of recombinant calbrain protein and its calcium binding property.** *Panel A*, the recombinant calbrain protein from plasmid expression system was purified by a nickel nitrilotriacetic acid resin column. 4 μg of pure CaM (*lane 1*), 3 μg of recombinant calbrain protein (*lane 2*), and control sample (proteins obtained from *E. coli* with nonrecombinant plasmid and purified by the same procedure) (*lane 3*) were run on a 10% Tricine SDS-PAGE gel. They were electrophoresed and stained with Coomassie Brilliant Blue. The duplicate of the electrophoresed gel was transferred to a nitrocellulose membrane and incubated with $^{45}Ca^{2+}$ and washed, then the membrane was exposed and analyzed by Fuji imaging system. The negative control sample (proteins obtained from *E. coli* with nonrecombinant plasmid) showed no signal. *M*, molecular mass markers. *Panel B*, the data was carried out by equilibrium dialysis. Conditions: 10 mM MOPS, pH 7.1, 150 mM KCl, 1.0 mM dithiothreitol, 0.1 mM EGTA, and 3 mM $MgCl_2$. *Inset*, Scatchard plot of data. *V*, mol of Ca^{2+} bound/mol of calbrain; *C*, free $[Ca^{2+}]$.

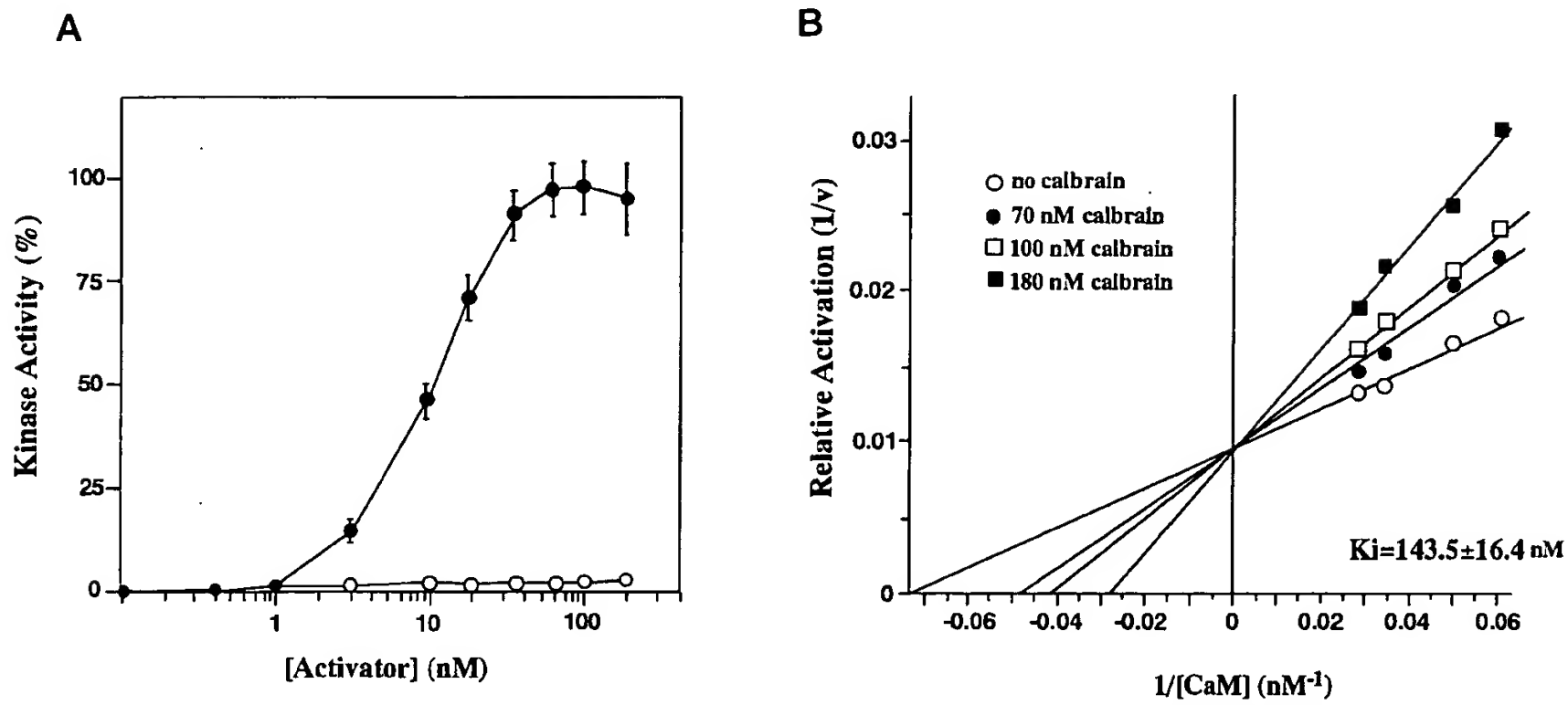

FIG. 6. **Comparison of *in vitro* activation of CaM-kinase II by calbrain and CaM.** *Panel A*, the activity of purified bovine CaM-kinase II in the presence of various concentrations of calbrain or CaM was assessed using syntide-2 as a substrate for phosphorylation. The kinase activity in the presence of 100 nM CaM was measured and designated as 100%. The relative ratio (%) of activities in the presence of various concentrations of calbrain (*open circles*) and CaM (*filled circles*) were then calculated. Data are represented as the means of triplicate determinations (±S.E.). *Panel B*, activation assays were performed in the presence of indicated concentrations of calbrain and varying concentrations of CaM. $1/v$ represents 1/activity of CaM-kinase II (%). Note that calbrain competitively inhibits CaM-kinase II activation by CaM. The results are the mean (±S.E.) for two successive experiments performed in duplicate.

presence of 3.0 mM $MgCl_2$ and 150 mM KCl, calbrain binds 2.0 mol of Ca^{2+}/mol of protein with an apparent K_d of 0.194 μM.

The Inhibitory Effect of Calbrain on CaM-kinase II Activity, Autophosphorylation, and CaM Binding—The effect of calbrain on CaM-kinase II activity is shown in Fig. 6, *A* and *B*. The activity of CaM-kinase II incubated in the solution containing 0–200 μM calbrain without CaM (Fig. 6*A*, *open circles*) revealed that calbrain was not able to activate CaM-kinase II. On the other hand, Fig. 6*B* showed that calbrain competitively inhibited activation of CaM-kinase II by CaM with a K_i value of 143.5 ± 16.4 nM (mean ±S.E.). The effect of calbrain on the autophosphorylation of CaM-kinase II was examined, and the results were shown in Fig. 7. Calbrain also competitively inhibited CaM-dependent autophosphorylation of CaM-kinase II with a K_i value of 189.7 ± 12.4 nM (mean ±S.E.), whereas calbrain itself did not induce autophosphorylation of CaM-kinase II (data not shown). The binding study of CaM and calbrain to CaM-kinase II revealed that calbrain competitively inhibited CaM binding to CaM-kinase II, and the K_i value of this inhibition was 128.6 ± 19.7 nM (mean ±S.E.) (Fig. 8).

DISCUSSION

In the present study, we have shown that two EF-hand motifs of calbrain, a novel Ca^{2+}-binding protein, have a significant homology to those of CaM and other related four EF-hand Ca^{2+}-binding proteins. The first EF-hand motif of calbrain is very similar to the first and the third motifs, and the second EF-hand motif of calbrain very similar to the second and the fourth motifs of CaM and troponin C. Although calbrain is a two EF-hand protein, the homology between calbrain and two EF-hand proteins such as S100β was found to be very low. From the high sequence homology between EF-hand motifs, it has been proposed that CaM, troponin C, and myosin light chain gene evolved from a common four-domain molecule (29, 31, 32). In each of these proteins, the first and the third and the second and fourth EF-hand domains show high homology, supporting the hypothesis that these proteins evolved from a two-domain precursor by gene duplication (33, 34). The significant homology between the EF-hand motifs of calbrain and CaM family suggests that although calbrain is a two EF-hand protein, evolutionally it is related closely to the CaM family rather than two EF-hand protein families. As calbrain is the first two EF-hand protein whose domains appear to be very similar to those of four EF-hand proteins, our findings may be interesting from the aspect of the evolution of EF-hand proteins.

The biological functions of EF-hand proteins are strongly related to the conformational changes of EF-hand domains in response to Ca^{2+} binding. EF-hand domains that show large conformational changes by binding Ca^{2+} are known to have a trigger function in the activation of target proteins. The domains that have regulatory roles are termed regulatory domains (35, 36), and proteins that have such domain(s) are called Ca^{2+} sensor proteins. For example, CaM and troponin C, which are classified in this category, enable the cell to detect a stimulatory influx of Ca^{2+} and thereby transduce this signal into a variety of cellular processes (37). On the other hand, EF-hand domains that exhibit small conformational changes are termed structural or buffer domains (36, 38). These domains are responsible for the structural stability, local Ca^{2+} transport, and function in buffering intracellular Ca^{2+}. Proteins possessing these domains, such as calbindin and parvalbumin, are called Ca^{2+} buffer proteins (39, 40). From the amino acid sequence in the present study, it is difficult to predict whether calbrain has regulatory or structural domains, and therefore is a Ca^{2+} sensor or Ca^{2+} buffer protein. It has been suggested that the interhelical angle changes of EF-hand proteins upon Ca^{2+} binding become a good index for their classification. Another useful method is to check whether or not the protein can bind to a hydrophobic column in a Ca^{2+}-dependent manner (36). Ca^{2+} sensor proteins that cause large conformational changes bind to the column by Ca^{2+}-induced exposure of the hydrophobic surface in the protein formed by a pair of EF-hands. After that, these proteins can be eluted by removing Ca^{2+} with EGTA. This method was successfully applied for the purification of CaM (41). We have purified the recombinant

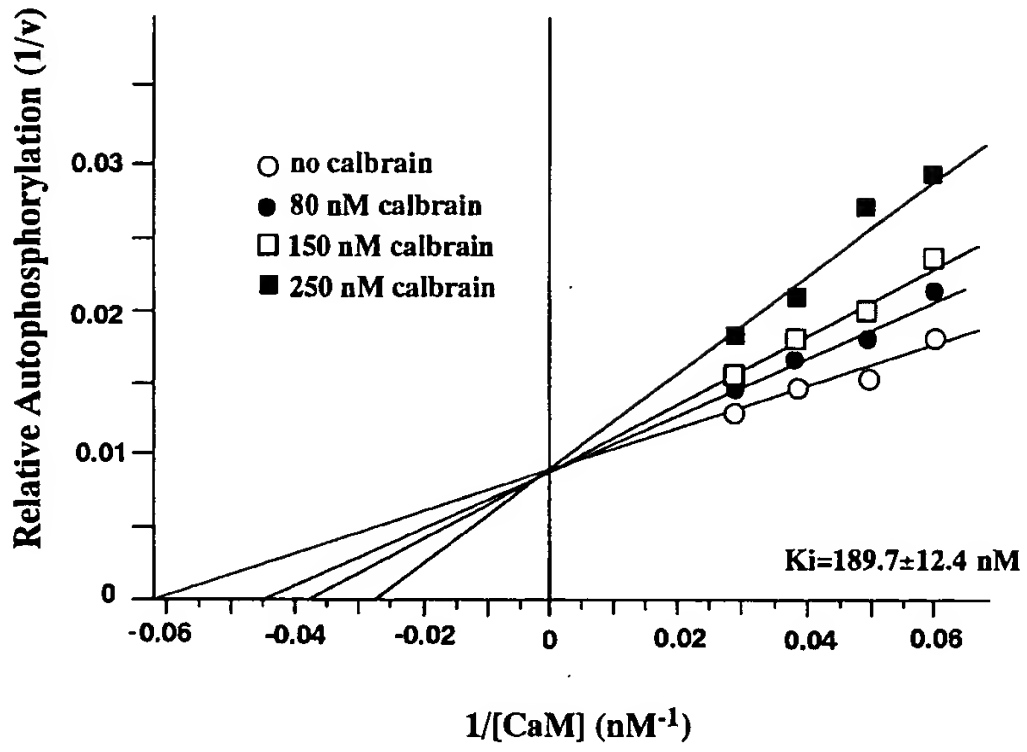

FIG. 7. **Inhibition of CaM-kinase II autophosphorylation by calbrain.** The autophosphorylation of purified bovine CaM-kinase II in the presence of indicated concentrations of calbrain and varying concentrations of CaM was assessed by measuring [γ-^{32}P]ATP radioactivity of CaM-kinase II extracted from electrophoresed gels. Autophosphorylation assays were performed as described under "Experimental Procedures." *1/v* represents 1/autophosphorylation of CaM-kinase II (%). Note that calbrain competitively inhibits CaM-dependent CaM-kinase II autophosphorylation. The results are the mean (±S.E.) for two successive experiments performed in duplicate.

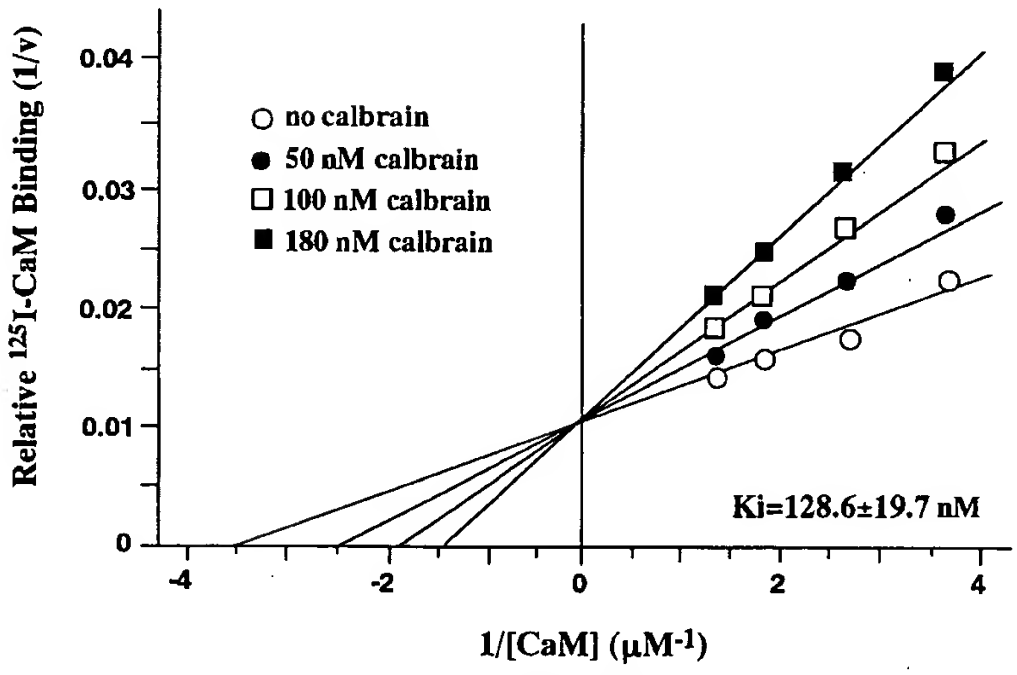

FIG. 8. **Competitive inhibition of CaM binding to CaM-kinase II by calbrain.** The binding of CaM to CaM-kinase II in the presence of indicated concentrations of calbrain was studied. ^{125}I-CaM and calbrain were mixed with CaM-kinase II and Ca^{2+} under nonautophosphorylated conditions (in ATP-free reaction mixture). CaM-kinase II was immunoprecipitated by anti-CaM-kinase II antibody after the reaction, and the radioactivity of each sample was measured. The radioactivity of the bound ^{125}I-CaM in the presence of 500 nM CaM and 0 nM calbrain was measured and designated as 100%. The relative ratio (%) of radioactivities of the samples were then calculated. *1/v* represents 1/CaM binding rate to CaM-kinase II (%). The results are the mean (±S.E.) for two successive experiments performed in duplicate.

calbrain protein by this method, also (data not shown), and the results indicate that calbrain possesses a regulatory domain that shows a large conformational change with Ca^{2+} binding and, therefore, can be classified as a Ca^{2+} sensor protein.

The distribution study of calbrain mRNA revealed that calbrain is a brain-specific Ca^{2+}-binding protein that is expressed abundantly in the hippocampus, in the habenular nucleus of the epithalamus and in the Purkinje cell layer of the cerebellum. The specific tissue distribution of EF-hand proteins has suggested their particular functions in each tissue (42–44). The localization of calbrain mRNA in the hippocampus and cerebellum, together with its functional potency as a Ca^{2+} sensor protein being involved in the Ca^{2+} signal transduction suggest an important role for this protein in the central nervous system. A number of studies on hippocampus have shown that this part of the brain is particularly involved in acquisition and storage of spatial information (45–47).

CaM-kinase II is a multifunctional serine/threonine protein kinase capable of phosphorylating several endogenous proteins in the brain (48, 49) and is highly expressed in the mammalian central nervous system (50–52). This enzyme is a major component of postsynaptic density (24, 53, 54) and plays important roles in the regulation of the neurotransmitter synthesis, receptor function, axonal transport, gene expression, and especially in the long-term potentiation, which is an established model of neural plasticity (14, 55, 56). Many biochemical studies have indicated that phosphorylation induced by CaM-kinase II can act as a molecular switch, conferring properties that are advantageous for long-lasting storage of changes initiated by brief Ca^{2+} signals (57–59). The binding study of CaM and calbrain to CaM-kinase II indicated that under nonautophosphorylated conditions, calbrain inhibited CaM binding to CaM-kinase II competitively (Fig. 8). Although calbrain could not activate CaM-kinase II, Fig. 6*B* showed that calbrain competitively inhibited the activation of CaM-kinase II by CaM. Because K_i values of binding and activity inhibition were similar, it was supposed that inhibition of CaM binding by calbrain may caused the reduction of kinase activity. CaM-kinase II activity is regulated by Ca^{2+}/CaM and autophosphorylation (60). When CaM binds to the CaM binding domain, a confor-

mational change is induced in the regulatory region, and the interaction of inhibitory domain with the active site is disrupted. It allows the active site to become accessible to exogenous substrate. When Ca^{2+}/CaM is bound, CaM-kinase II is rapidly autophosphorylated on Thr^{286}, and autophosphorylation increases CaM binding affinity to the kinase dramatically by decreasing of CaM-releasing time (61). Under both experimental conditions used for the kinase activity assay (incubation at 30 °C for 1 min) and autophosphorylation assay (at 0 °C for 10 min), autophosphorylation on Thr^{286} of CaM-bound kinase occurs (24–26). Therefore, the activity detected in Fig. 6*B* may be influenced by a change of CaM affinity caused by autophosphorylation. K_i values of kinase activity (143.5 ± 16.4 nM) and autophosphorylation (189.7 ± 12.4 nM) did not significantly differ from that of CaM binding (128.6 ± 19.7 nM) under nonautophosphorylated condition.

This is the first report of a novel Ca^{2+}-binding protein that can inhibit CaM-dependent CaM-kinase II activity. Although calbrain can reduce autophosphorylation, it is supposed that this protein is involved in the physiological regulation of CaM-kinase II. As CaM-kinase II has multiple functions and essential roles in the brain, calbrain may also play important roles in the central nerve system.

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REFERENCES

1. Carafoli, E. (1987) *Annu. Rev. Biochem.* **56,** 395–433
2. Siesjö, B. K., and Wieloch, T. (1986) *Adv. Neurol.* **44,** 813–847
3. Rubin, R. P. (1970) *Pharmacol. Rev.* **22,** 389–428
4. Rasmussen, H., and Goodman, D. B. P. (1977) *Physiol. Rev.* **57,** 421–509
5. Ghosh, A., and Greenberg, M. E. (1995) *Science* **14,** 239–247
6. Delorenzo, R. J., Freedman, S. D., Yohe, W. B., and Maurer, S. C. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 1838–1842
7. Delorenzo, R. J. (1981) *Cell Calcium* **2,** 365–385
8. Davis, T. N. (1992) *Cell* **71,** 557–564
9. Kretsinger, R. H. (1976) *Annu. Rev. Biochem.* **45,** 239–266
10. Kawasaki, H., and Kretsinger, R. H. (1994) *Protein Profile* **1,** 343–517
11. Vanaman, T. C. (1980) in *Calcium and Cell Function*, (Cheung, W. Y., ed) Vol. 1, pp. 41–58, Academic Press Inc., New York
12. GuptaRoy, B., Beckingham, K., and Griffith, L. C. (1996) *J. Biol. Chem.* **271,** 19846–19851
13. Shields, S. M., Vernon, P. J., and Kelly P. T. (1984) *J. Neurochem.* **43,** 1599–1609
14. Hanson, P. I., and Schulman, H. (1992) *Annu. Rev. Biochem.* **61,** 559–601
15. Hanson, P. I., Meyer, T., Stryer, L., and Schulman, H. (1994) *Neuron* **12,** 943–956
16. Hagiwara, T., Ohsako, S., and Yamauchi, T. (1991) *J. Biol. Chem.* **266,** 16401–16408
17. Mukherji, S., and Soderling, T. R. (1994) *J. Biol. Chem.* **269,** 13744–13747
18. Sanger, F., Nicklen, S., and Coulson, A. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 5463–5467
19. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol.* **215,** 403–410
20. Schägger, H., and von Jago, W. G. (1987) *Anal. Biochem.* **199,** 223–231
21. Kyhase-Andersen, J. (1984) *J. Biochem. Biophys. Methods* **10,** 203–209
22. Deleted in proof
23. Fabiato, A. (1981) *J. Gen. Physiol.* **78,** 457–497
24. Ochiishi, T., Sugiura, H., and Yamauchi, T. (1993) *Brain Res.* **610,** 97–107
25. Yamauchi, T., and Fujisawa, H. (1980) *FEBS Lett.* **116,** 141–144
26. Yoshimura, Y., and Yamauchi, T. (1997) *J. Biol. Chem.* **272,** 26354–26359
27. Hunter, T., and Sefton, B. M. (1991) *Methods in Enzymology* (Hunter, T., and Setton, B. M., eds), pp. 274–283, Academic Press, San Diego, CA
28. Segel, I. H. (1975) *Enzyme Kinetics* (Bergmeyer, H.), John Wiley & Sons, Inc., New York
29. Kretsinger, R. H. (1980) *Crit. Rev. Biochem.* **8,** 119–174
30. Scatchard, G. (1949) *Ann. N. Y. Acad. Sci.* **51,** 660–672
31. Goodman, M., Pechère, J. F. Haiech, J., and Demaille, J. G. (1979) *J. Mol. Evol.* **13,** 331–352
32. Fischer, R., Koller, M., Flura, Mathews, S., Strehler-Page, M. A., Krebs, J., Penniston, J. T., Carafoli, E., and Strehler, E. E. (1988) *J. Biol. Chem.* **263,** 17055–17062
33. Perret, C. (1988) *J. Mol. Evol.* **27,** 351–364
34. Parmentier, M., Lawson, D. E. M., and Vassart, G. (1987) *Eur. J. Biochem.* **170,** 207–215
35. Klee, C. B., Draetta, G. F., and Hubbard, M. J. (1988) *Adv. Enzymol. Relat. Areas Mol. Biol.* **61,** 149–200
36. Ikura, M. (1996) *Trends Biochem. Sci.* **21,** 14–17
37. deSilva, J. J. R., and Williams, R. J. P. (1991) *The Biological Chemistry of the Elements* (de Silva, J. J. R., and Williams, R. J. P., eds), pp. 268–298, Oxford University Press, London
38. Baimbridge. K. G., Miller, J. J., and Parkes, C. O. (1982) *Brain Res.* **239,** 519–525
39. Mattson, M. P., Rychlik, B., Chu, C., and Christakos, S. (1991) *Neuron* **6,** 41–51
40. Kosaka, T., Kosaka, K., Nakayama, T., Hunziker, W., and Heizmann, C. W. (1993) *Exp. Brain Res.* **93,** 483–491
41. Gophalakrishna, R., and Anderson, W. B. (1982) *Biochem. Biophys. Res. Commun.* **104,** 830–836
42. Braun, K. (1990) *Prog. Histochem. Cytochem.* **21,** 1–64
43. Baimbridge, K. G., Celio, M. R., and Rogers, J. H. (1992) *Trends Neurosci.* **15,** 303–308
44. Andressen, C., Blümcke, I., and Celio, M. R. (1993) *Cell Tissue Res.* **271,** 181–208
45. McNaughton, B. L., and Morris, R. G. M. (1987) *Trends Neurosci.* **10,** 408–415
46. Morris, R. G. M., Garrud, P., Rawlins, J. N. P., and O'Keefe, J. (1982) *Nature* **297,** 681–683
47. Olton, D. S., Walker, J. A., and Gage, F. H. (1978) *Brain Res.* **139,** 295–308
48. Cheung, W. Y. (1980) *Science* **207,** 19–27
49. Reichardt, L. F., and Kelly, R. B. (1983) *Annu. Rev. Biochem.* **52,** 871–926
50. Bennett, M. K., Erondu, N. E., and Kennedy, M. B. (1983) *J. Biol. Chem.* **258,** 12735–12744
51. Yamauchi, T., Ohsako, S., and Deguchi, T. (1989) *J. Biol. Chem.* **264,** 19108–19116
52. Yamauchi, T., Sekihara, S., and Ohsako, S. (1991) *Brain Res.* **541,** 198–205
53. Goldenring, J. R., McGuire, J. S., Jr., and Delorenzo, R. J. (1984) *J. Neurochem.* **42,** 1077–1084
54. Kelly, P. T. McGuinness, T. L., and Greengard, P. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 945–949
55. Colbran, R. J., Schworer, C. M., Hashimoto, Y., Fong, Y. L., Rich, D. P., Smith, M. K., and Soderling, T. R. (1980) *Biochem. J.* **258,** 313–325
56. Lisman, J., Malenka, R. C., Nicoll, R. A., and Malinow, R. (1997) *Science* **276,** 2001–2002
57. Lisman, J. E. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 3055–3057
58. Lisman, J. E. (1993) *Trends Neurosci.* **17,** 406–412
59. Miller, S. G., and Kennedy, M. B. (1986) *Cell* **44,** 861–870
60. Colbran, R. J., Fong, Y. L., Schworer, C. M., and Soderling, T. R. (1988) *J. Biol. Chem.* **263,** 18145–18151
61. Meyer, T., Hanson, P. I., Stryer, L., and Schulman, H. (1992) *Science* **256,** 1199–1202

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Primary Structure and Binding Properties of Calgranulin C, a Novel S100-like Calcium-binding Protein from Pig Granulocytes*

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In this paper we report the biochemical characterization of calgranulin C, a new member of the S100 protein family. The protein is highly abundant in the cytosol of pig granulocytes, with relatively small amounts in lymphocytes. A simple protocol for the rapid purification of calgranulin C is described. The purified protein migrates as a single entity on SDS-polyacrylamide gel electrophoresis while it has two isoforms focusing at pH 5.8 and 5.5. Gel filtration and cross-linking experiments indicate that calgranulin C is capable of dimerization. The complete amino acid sequence was determined by Edman degradation of peptides generated by trypsin and V8 protease digestion. Calgranulin C consists of 91 residues and has a calculated molecular mass of 10,614 daltons. This value is virtually identical to that obtained by electrospray mass spectrometry. Sequence analysis predicts two EF-hand calcium-binding motifs, the first having an extended loop that is distinctive of the S100 protein family. The metal-binding properties were studied by means of a direct $^{45}Ca^{2+}$-binding assay and by tyrosine fluorescence titration. Calgranulin C binds not only calcium but also zinc ions. A single high affinity Zn^{2+}-binding site per monomer was evidenced by fluorimetric titration. Zinc binding to calgranulin C induces a remarkable increase in the protein affinity for calcium; in the absence of zinc, the protein binds 1 Ca^{2+}/monomer with a binding constant of about 2×10^4 M^{-1}, whereas the Zn^{2+}-loaded form binds 2 Ca^{2+}/monomer with K_a values of approximately 3×10^7 and 6×10^4 M^{-1}. Circular dichroism analysis showed that the binding of calcium to calgranulin C induces a 15% decrease in the apparent α-helix content. This result and the calcium-dependent binding of the protein to a phenyl-Superose column strongly suggest that calgranulin C undergoes a gross conformational change upon calcium binding, thus supporting the idea that this protein may be involved in Ca^{2+}-dependent signal transduction events.

Intracellular Ca^{2+} is a ubiquitous second messenger involved in the regulation of many cellular functions (1). The signal is partly transduced into metabolic or mechanical responses by calcium-binding proteins (CaBPs)[1] that interact with cellular effectors in a Ca^{2+}-dependent fashion (2). These proteins include Ca^{2+}/phospholipid-binding proteins of the annexin family (3) and EF-hand CaBPs such as calmodulin, troponin C, and a number of S100 proteins (4, 5). The function of calmodulin in Ca^{2+} signal transduction has been studied extensively, and many target enzymes have been identified (reviewed in Ref. 6). Calcium binding to calmodulin induces a conformational change, thus exposing hydrophobic sites that are involved in the interaction with target proteins (7, 8). The fact that other EF-hand CaBPs also expose hydrophobic regions upon calcium binding (5, 9–12) suggests that this model may represent a general mechanism for the function of these proteins as Ca^{2+} signal mediators.

In granulocytes and monocytes, intracellular Ca^{2+} regulates various acute response activities such as the respiratory burst, phagocytosis, degranulation, and chemotaxis (13–16). Regional increases in Ca^{2+} are thought to occur at sites within the cell where these activities take place (16). Recent efforts to identify calcium signal mediators in granulocytes have led to the discovery of new CaBPs, namely a 33-kDa annexin (17) and a 28-kDa EF-hand protein named grancalcin (18). Additionally, these cells express calmodulin (19) and a heterocomplex formed by two S100 proteins, calgranulins A and B (20, 21).[2]

We have previously reported a preliminary characterization of two abundant CaBPs from pig granulocytes (22). N-terminal sequencing suggested that both proteins belong to the S100 protein family. We identified one of these CaBPs as the porcine counterpart of calgranulin A (22) and proved that, as described for the human and bovine systems (23, 24), it is noncovalently associated with pig calgranulin B (25). Here we focus on the characterization of the other CaBP and demonstrate that it is a new member of the S100 protein family. Its primary structure as well as some binding properties are described. This protein will be referred to as calgranulin C, consistent with the names adopted for other S100 proteins isolated from granulocytes (21, 26).

EXPERIMENTAL PROCEDURES

Materials—$^{45}CaCl_2$ (5 Ci/g) was from Du Pont NEN. Electrophoresis reagents were purchased from Bio-Rad. Dimethyl suberimidate was from Pierce. Sequencing-grade reagents and solvents were obtained from Applied Biosystems. Sequelon-AA™ membranes were from Millipore Corp. PhastGel 4–6.5, molecular mass markers, and pI markers were from Pharmacia LKB (Uppsala, Sweden). Percoll, Sephadex G-75, horse heart myoglobin, bovine serum albumin, trypsin, and V8 protease were purchased from Sigma. All other solvents and reagents were of analytical grade.

Preparation of Lymphocyte and Granulocyte Extracts—Pig lymphocytes and granulocytes were isolated from fresh blood by dextran

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The amino acid sequence reported in this paper has been submitted to the Protein Identification Resource and SWISS-PROT protein sequence data banks with the accession number P80310.

[1] The abbreviations used are: CaBP, calcium-binding protein; ESMS, electrospray mass spectrometry; FPLC, fast protein liquid chromatography; HPLC, high performance liquid chromatography; RP-HPLC, reverse phase-HPLC; PAGE, polyacrylamide gel electrophoresis; V8 protease, Glu-C-specific endoprotease from *Staphylococcus aureus* strain V8; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; Tricine, *N*-tris(hydroxymethyl)methylglycine.

[2] Synonyms of calgranulins A and B are MRP8 and MRP14, L1 light and heavy chain, p8 and p14, and p7A and p24, respectively.

sedimentation followed by Percoll gradient centrifugation (22) and were more than 95% pure. Cells were suspended in homogenization buffer (10 mM phosphate, 150 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, pH 7.0), and disrupted in a glass-Teflon homogenizer. The homogenate was centrifuged at 30,000 × *g* for 15 min at 4 °C. The resulting supernatant was centrifuged at 105,000 × *g* for 90 min at 4 °C.

Purification of Calgranulin C—Supernatants from cell extracts (3–4 ml) were fractionated at 4 °C on a Sephadex G-75 column (2.5 × 38 cm) equilibrated with the homogenization buffer. Elution was performed at a flow rate of 15 ml/h. Eluted fractions corresponding to a molecular mass of 10–15 kDa (elution volume: 125–140 ml) were pooled, diluted 3-fold with 20 mM Tris-HCl (pH 9.0), and loaded onto a Mono Q HR 5/5 column (Pharmacia LKB) previously equilibrated with the same buffer. The column was developed on an FPLC system (Pharmacia LKB) with a combination of two linear gradients of NaCl concentration (0–0.14 M in 20 min followed by 0.14–0.35 M in 15 min). The flow rate was 1 ml/min. Calgranulin C eluted at approximately 0.15 M NaCl.

Protein Assay—Protein concentration in crude cell extracts was estimated by Lowry's method (27) with bovine serum albumin as a standard. The concentration of pure calgranulin C was determined by UV absorbance in 6 M guanidine-HCl (28). As the protein has 2 tyrosine residues and no tryptophan (see "Results"), an $\epsilon_{275\ nm}$ = 2900 M^{-1} cm^{-1} was used (29).

Electrophoresis—SDS-PAGE was performed as described by Schägger and von Jagow (30) in a Mini-PROTEAN II apparatus (Bio-Rad). Isoelectric focusing was carried out in a Phast System (Pharmacia LKB). Densitometric analysis of Coomassie-stained gels was performed in a Dual-Wavelength Chromato Scanner (Shimadzu, Kyoto, Japan).

Cross-linking Experiments—Protein samples in 0.2 M Bicine (pH 8.5) were treated with 1 mM dimethyl suberimidate at 20 °C during 1 h. Subsequently, the reaction was quenched by the addition of glycine to a final concentration of 10 mM, and reaction products were analyzed by SDS-PAGE.

Chromatographic Analysis—Gel filtration analysis of pure calgranulin C (10 μg) was performed by FPLC on a Superose 12 HR 10/30 column (Pharmacia LKB) calibrated with standard proteins. The column was eluted with 50 mM Tris-HCl (pH 7.4) at a flow rate of 0.5 ml/min.

Hydrophobic interaction chromatography was carried out on a phenyl-Superose HR 5/5 column (Pharmacia LKB) equilibrated with 50 mM Tris-HCl (pH 7.4), 0.5 mM $CaCl_2$. After the injection of 30 μg of pure protein, the column was eluted with four column volumes of the same buffer and then with four column volumes of 50 mM Tris-HCl, 1 mM EDTA (pH 7.4).

Mass Spectrometry—The molecular mass of calgranulin C was determined on a VG BioTech/Fisons (Altrincham, United Kingdom) triple-quadrupole instrument equipped with an electrospray ionization source (Analytica). The sample was injected into the ion source in 50% (v/v) methanol and 1% (v/v) acetic acid. Fifteen scans ranging from m/z 800 to 1600 were recorded in each determination. The instrument was calibrated with horse heart myoglobin (average mass 16,951.5 Da).

Enzymatic Digestion and Peptide Purification—The purified protein (400 μg) was digested in 0.1 M ammonium bicarbonate (pH 7.8) with 4 μg of trypsin or 6 μg of V8 protease, at 37 °C during 24 h. Peptides were fractionated by HPLC (Pharmacia LKB) on a Vydac C_{18} column (4.6 × 250 mm) equilibrated with 0.1% (v/v) trifluoroacetic acid in water. Elution was performed at a flow rate of 0.8 ml/min with a 0–80% acetonitrile linear gradient in 100 min.

Amino Acid Analysis and Sequencing—Amino acid analysis was performed on a model 420A amino acid analyzer (Applied Biosystems). Amino acid sequencing was carried out on an Applied Biosystems model 477A protein sequencer equipped with an on-line model 120A phenylthiohydantoin analyzer. The intact protein, as well as most of the peptides, was loaded onto a Polybrene-coated glass filter and sequenced according to the manufacturer's instructions. Peptides V7–V10 were covalently bound to a Sequelon-AA™ membrane. In this case, sequences were run basically as described by Admon and King (31).

Sequence Comparison—Multiple sequence alignment and phylogenetic tree construction were carried out by using the Darwin system (32).

Calcium Binding Assay—Apo-calgranulin C was prepared by incubation of freshly purified protein with 2 mM EGTA and 2 mM EDTA and subsequent dialysis against 25 mM Tris-HCl (pH 7.4), or by extensive dialysis against 10 mM EDTA (pH 7.4) and then against Milli Q water (Millipore Corp.) (33).

$^{45}Ca^{2+}$ binding was determined as per the method of Mani and Kay (11) that uses microconcentrators as ultrafiltration devices to perform rapid flow dialysis. In short, apo-calgranulin C (15–50 μM) was

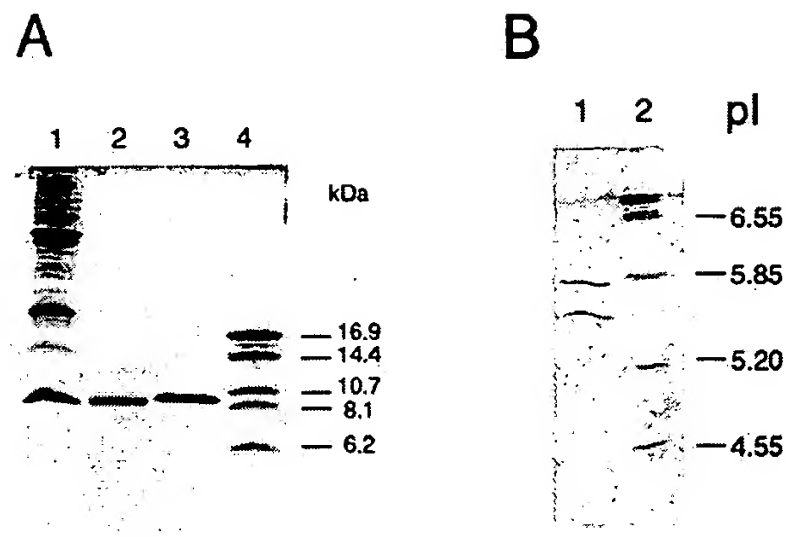

FIG. 1. *A*, SDS-PAGE analysis of calgranulin C-containing samples at different stages of purification. The purification procedure is described under "Experimental Procedures." *Lane 1*, 105,000 × *g* supernatant from pig granulocytes; *lane 2*, after Sephadex G-75 gel filtration; *lane 3*, after Mono Q chromatography; *lane 4*, molecular mass standards. *B*, isoelectric focusing of purified calgranulin C on a PhastGel IEF 4–6.5. *Lane 1*, calgranulin C; *lane 2*, pI standards.

incubated in a prewashed Centricon 3 microconcentrator (Amicon) with known amounts of $^{45}CaCl_2$ at 20 °C during 5 min and then the sample was centrifuged at 3000 rpm for 5 min. Free $^{45}Ca^{2+}$ concentration was determined by measuring the radioactivity in the filtrate. Each determination was performed at least in triplicate.

Binding data were analyzed by means of the following equations, where ν is the number of moles of calcium bound per mol of monomer, X is free calcium concentration, n is the number of binding sites per monomer, and K_a, K_{a_1}, and K_{a_2} are macroscopic binding constants.

$$\nu = \frac{n K_a x}{1 + K_a x} \qquad \text{(Eq. 1)}$$

$$\nu = \frac{\frac{n}{2} K_{a_1} x + n K_{a_1} K_{a_2} x^2}{1 + K_{a_1} x + K_{a_1} K_{a_2} x^2} \qquad \text{(Eq. 2)}$$

Curve-fitting was made by nonlinear regression (SigmaPlot 4.10, Jandel Corp.). The calculated parameters are expressed as mean ± S. E.

Fluorescence Measurements—Tyrosine fluorescence was registered at 25 °C on a Jasco FP-770 spectrofluorometer (Japan Spectroscopic Co., Hachioji City, Japan). The excitation wavelength was set to 278 ± 5 nm. Each spectrum represents an average of five scans. For titration experiments, the emission wavelength was set to 308 ± 3 nm. The fluorescence intensity (F) was corrected for sample dilution, the latter never exceeding 3%. Data from titration with calcium in the absence of zinc were fitted to Equation 3, where F_o is the fluorescence at zero ligand concentration, F_m is the maximum fluorescence change, T is the total ligand concentration, P is the protein monomer concentration, and K_a is the apparent association constant.

$$F = F_o + \frac{F_m}{2P}\left(T + P + \frac{1}{K_a} - \sqrt{\left(T + P + \frac{1}{K_a}\right)^2 - 4PT}\right) \qquad \text{(Eq. 3)}$$

This equation is derived from Eq. 1 by setting n to 1 and substituting $[(F - F_o)\cdot F_m^{-1}]$ for ν and $[T - P\cdot F_m^{-1}\cdot(F - F_o)]$ for X.

Circular Dichroism—Far-UV circular dichroism spectra were obtained on a Jasco J-20 spectropolarimeter calibrated with (+)-10-camphorsulfonic acid. Each spectrum represents an average of four scans. The α-helix content was calculated as described by Zhong and Johnson (34).

RESULTS

Purification of Calgranulin C—The 105,000 × *g* supernatant from pig granulocytes was fractionated on a Sephadex G-75 column. Calgranulin C was recovered as a major component in the 10–15-kDa fraction (Fig. 1*A*, *lane 2*) and was further purified by anion exchange on a Mono Q column. The protein eluted from the column in a symmetric peak and was considered homogeneous per SDS-PAGE (Fig. 1*A*, *lane 3*) and N-terminal sequence. Calgranulin C could also be purified from lympho-

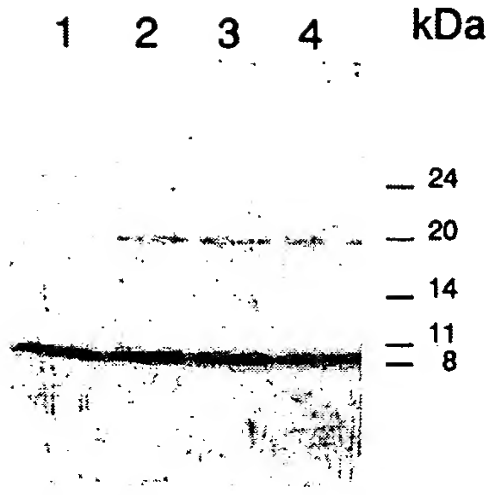

FIG. 2. **Dimer formation of calgranulin C *in vitro*.** The purified protein was analyzed by 16% SDS-PAGE without cross-linking (*lane 1*) and after cross-linking with 1 mM dimethyl suberimidate in the presence of 1 mM EDTA (*lane 2*), 1 mM $CaCl_2$ (*lane 3*), and 0.1 mM $ZnCl_2$ (*lane 4*).

cyte extracts by the same procedure, and its electrophoretic mobility, UV absorption spectrum, and N-terminal sequence (6 cycles) were indistinguishable from those of the protein isolated from granulocytes (data not shown). However, the amounts of calgranulin C obtained from the granulocyte and lymphocyte extracts were 8% and 0.14%, respectively of the total soluble proteins. Thus, the content of calgranulin C was 50–60 times higher in granulocyte than in lymphocyte extracts.

Biochemical Properties of Calgranulin C—The protein migrates on Tricine SDS-PAGE as a 9-kDa polypeptide (Fig. 1A, *lane 3*). As some calcium-binding proteins have aberrant mobilities on SDS-PAGE gels (18, 24, 35), a more precise determination of the molecular mass of calgranulin C was made by ESMS. The mass spectrum showed the presence of two components of 10,614 ± 3 and 10,654 ± 3 Da (mean ± S.D.), the second accounting for about 20% of the molecules. This difference in mass (40 Da) could correspond to a calcium atom bound to the molecule, although alternative explanations such as partial N-terminal acetylation (42 Da) should not be ruled out.

Analysis of the purified protein by Superose 12 gel filtration showed two peaks of an apparent molecular mass of 11 and 18 kDa, thus suggesting that the native protein exists both as a monomer and as a homodimer. This conclusion was further supported by cross-linking experiments. Upon treatment with dimethyl suberimidate and subsequent SDS-PAGE analysis, a new protein band of 20 kDa was observed in addition to that of the remaining monomer (Fig. 2, *lane 2*). The presence of either 1 mM $CaCl_2$ or 0.1 mM $ZnCl_2$ during the cross-linking reaction did not modify the dimer/monomer ratio (Fig. 2).

Two calgranulin C forms of pI 5.8 and 5.5 were observed by native isoelectric focusing (Fig. 1*B*). As inferred from densitometric scanning, the relative contents of pI 5.8 and 5.5 isoforms are about 75% and 25%, respectively. This proportion remained unchanged in samples incubated with either 2 mM $CaCl_2$ or 2 mM EDTA before isoelectric focusing. In addition, both forms were observed by denaturing isoelectric focusing (data not shown). Therefore, the charge heterogeneity of calgranulin C is not a consequence of ligand binding.

Primary Structure of Calgranulin C—The purified protein was desalted by RP-HPLC and submitted to N-terminal sequencing. The sequence of the first 40 residues was obtained, except for amino acids at positions 33 and 38, which could not be unambiguously identified. In order to complete the amino acid sequencing of calgranulin C, fragments were generated by enzymatic cleavage of the purified protein. Both tryptic and V8 protease peptides were separated by RP-HPLC and subsequently submitted to amino acid analysis and/or Edman degradation.

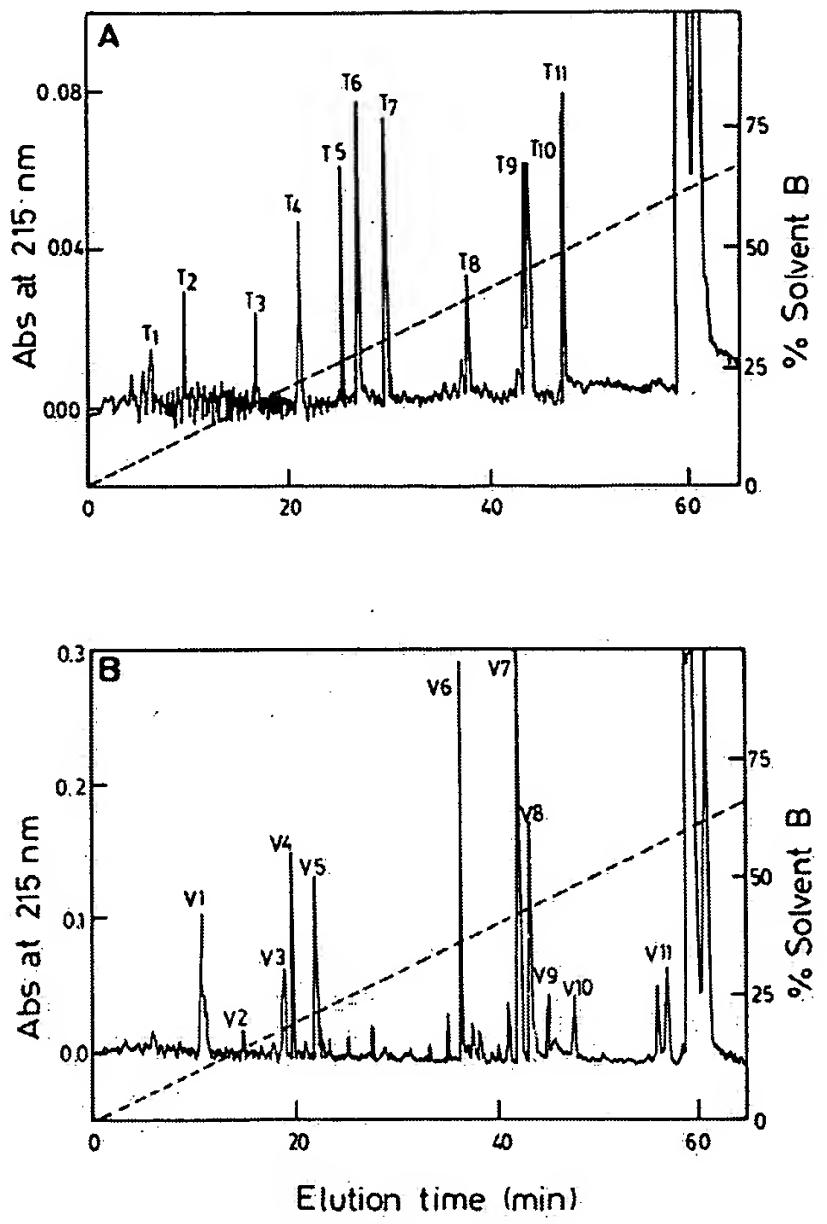

FIG. 3. **HPLC separation of peptides obtained by tryptic (*A*) and V8 protease (*B*) digestion of calgranulin C.** Details of the procedure are described under "Experimental Procedures." *Peaks T1–T11* and *V1–V11* represent peptides whose sequence was determined by Edman degradation or inferred from amino acid analysis. Solvent A: 0.1% trifluoroacetic acid; solvent B: 80% acetonitrile, 0.1% trifluoroacetic acid. No peaks were detected beyond 60% of solvent B.

The tryptic map of calgranulin C is shown in Fig. 3A. The peaks identified in the figure (designated T1–T11) represent pure peptides. Except for T6, T8, and T10, their amino acid sequences were determined by Edman degradation. On the basis of their amino acid composition, peptides T6 and T10 were assigned to fragments 21–29 and 1–20, respectively. The sequence of peptide T8 was partially obtained by Edman degradation, the rest being inferred from amino acid analysis. Considering the specificity of trypsin, it should be mentioned that the C-terminal residues of T9 and T11 were Tyr and Glu, respectively, instead of Arg or Lys as expected; the sequence of T9 was identical to that of residues 1–17 and was assumed to originate from a residual chymotryptic activity, while peptide T11 was assigned to the C-terminal end of the protein.

Fig. 3*B* shows the HPLC separation of fragments obtained by digestion with V8 protease. Again, the peaks identified in the figure represent pure peptides. Except for V6, V9, and V11, they were completely sequenced by Edman degradation.

A summary of the sequence analyses and the resulting primary structure of calgranulin C are shown in Fig. 4. The following peptides, all corroborating the proposed sequence, were not included in the figure (residue positions given in parentheses): T6 (21–29), T10 (1–20), V1 (5–8), V2 (1–4), and V9 (5–31). The protein consists of 91 residues and lacks cysteine, methionine, and tryptophan. On the basis of this sequence, the

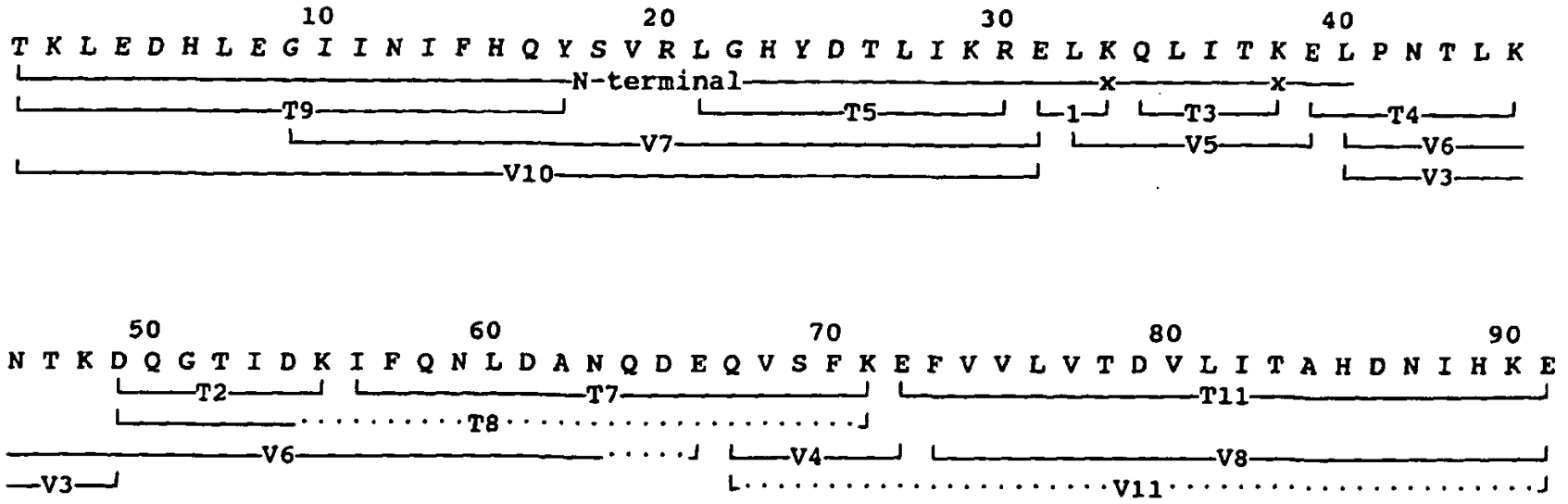

FIG. 4. **Primary structure of calgranulin C.** The nomenclature of peptides is consistent with that of Fig. 3 (*A* and *B*). *X* represents a residue which could not be identified on this particular run. Segments determined by Edman degradation or inferred from amino acid analysis are indicated with *solid* and *dotted lines*, respectively.

molecular mass was calculated to be 10,614 daltons. This value is virtually identical to that obtained by ESMS, thus indicating that the protein has been fully sequenced. The calculated isoelectric point (6.0) agrees with that obtained experimentally for the major calgranulin C isoform (5.8). The amino acid sequence predicts two EF-hand calcium-binding sites (36). The N-terminal site has a 14-residue loop (Ser^{18}–Glu^{31}) that is unique for S100 proteins, while the loop of the C-terminal EF-hand comprises 12 residues (Asp^{61}–Glu^{72}). The hydropathy plot (37) of calgranulin C predicts two hydrophobic regions near the N- and C-terminal ends (data not shown), a feature shared by the S100 proteins (4).

Direct Calcium Binding Studies—The Ca^{2+}-binding isotherm at 20 °C of calgranulin C in 25 mM Tris-HCl (pH 7.4) is shown in Fig. 5 (*closed circles*). Only one Ca^{2+}-binding site per monomer was titrated with free calcium concentrations of up to 0.6 mM. The best fit of the binding data with Equation 1 was obtained with $K_a = 1.9 \pm 0.4 \times 10^4\ M^{-1}$ and $n = 1.10 \pm 0.06$. Due to the experimental limitations of the method at higher ligand concentrations, the existence of an additional low affinity site ($K_a < 10^3\ M^{-1}$) could be neither proven nor ruled out.

As shown in Fig. 5, the presence of zinc ions induces a remarkable increase in the calgranulin C affinity for calcium. The binding isotherm obtained in the presence of 0.1 mM $ZnCl_2$ (*open circles*) was fitted to Equation 2 with the following parameters: $K_{a_1} = 2.7 \pm 0.3 \times 10^7\ M^{-1}$, $K_{a_2} = 6.5 \pm 1.2 \times 10^4\ M^{-1}$, and $n = 2.10 \pm 0.04$.

Tyrosine Fluorescence Titration—The intrinsic emission spectrum of apo-calgranulin C and those of the protein with Ca^{2+}, Mg^{2+}, and Zn^{2+} are shown in Fig. 6*A*. While tyrosine fluorescence was minimally influenced by 5 mM Mg^{2+}, a 7% decrease and a 40% increase were observed upon addition of 2 mM Ca^{2+} and 0.1 mM Zn^{2+}, respectively. Titration with Ca^{2+} in the absence of other metal ions showed the presence of a single class of site with $K_{a(app)} = 2.9 \pm 0.4 \times 10^4\ M^{-1}$ (Fig. 6*B*, *closed circles*), in agreement with direct binding experiments. The presence of 5 mM Mg^{2+} (*open circles*) caused a very slight decrease in the affinity for Ca^{2+} ($K_{a(app)} = 2.3 \pm 0.3 \times 10^4\ M^{-1}$), thus suggesting that the titrated site is highly selective for calcium.

A biphasic curve was obtained when calgranulin C was titrated with Ca^{2+} in the presence of 0.1 mM Zn^{2+} (Fig. 6*C*, *closed diamonds*). The curve was not noticeably influenced by 5 mM Mg^{2+} (*open diamonds*). Analysis of the Ca^{2+}-induced fluorescence change as a function of the fractional Ca^{2+} occupancy (νCa^{2+}), calculated with the previously determined binding constants, indicates that both the increase and decrease in tyrosine fluorescence are concomitant with the binding of calcium to the high and low affinity sites, respectively.

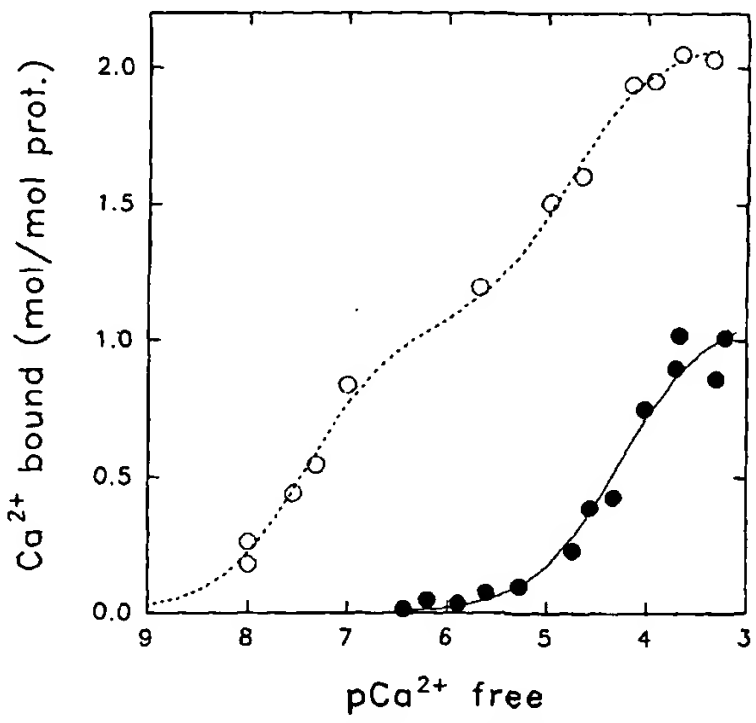

FIG. 5. **Ca^{2+}-binding isotherms of calgranulin C in the absence (*closed circles*) and in the presence (*open circles*) of 0.1 mM Zn^{2+}.** Details of the procedure are described under "Experimental Procedures." The curves depicted by *solid* and *dashed lines* were calculated by fitting Equations 1 and 2 to the experimental points, respectively. Values for the parameters are indicated in the text.

Fluorescence titration was also applied to study the binding of zinc to calgranulin C. As shown in Fig. 6*D*, fluorescence intensity increased almost linearly with added Zn^{2+} up to a ligand/protein monomer molar ratio of 1.0 ± 0.1, thus suggesting the presence of one high affinity Zn^{2+}-binding site per calgranulin C monomer. An almost identical titration curve was obtained in the presence of 0.1 mM Ca^{2+} (data not shown). Although the binding constant for Zn^{2+} could not be accurately calculated from these titration curves, simulations of the experimental data by means of Equation 3 (not shown) indicate that $K_{a(app)}$ should exceed $10^8\ M^{-1}$.

Calcium-induced Conformational Changes—Typical far-UV CD spectra of the apo- and Ca^{2+}-loaded forms of calgranulin C are shown in Fig. 7. The calcium-induced change in the CD spectrum may be attributed to a decrease in the overall α-helix content. Analysis of CD data according to Zhong and Johnson (34) indicated apparent α-helix contents of 52% and 44% for the apo- and Ca^{2+}-loaded forms, respectively.

Calcium binding also affected the chromatographic behavior of calgranulin C on a phenyl-Superose column. The protein was entirely bound to the column in the presence of 0.5 mM $CaCl_2$

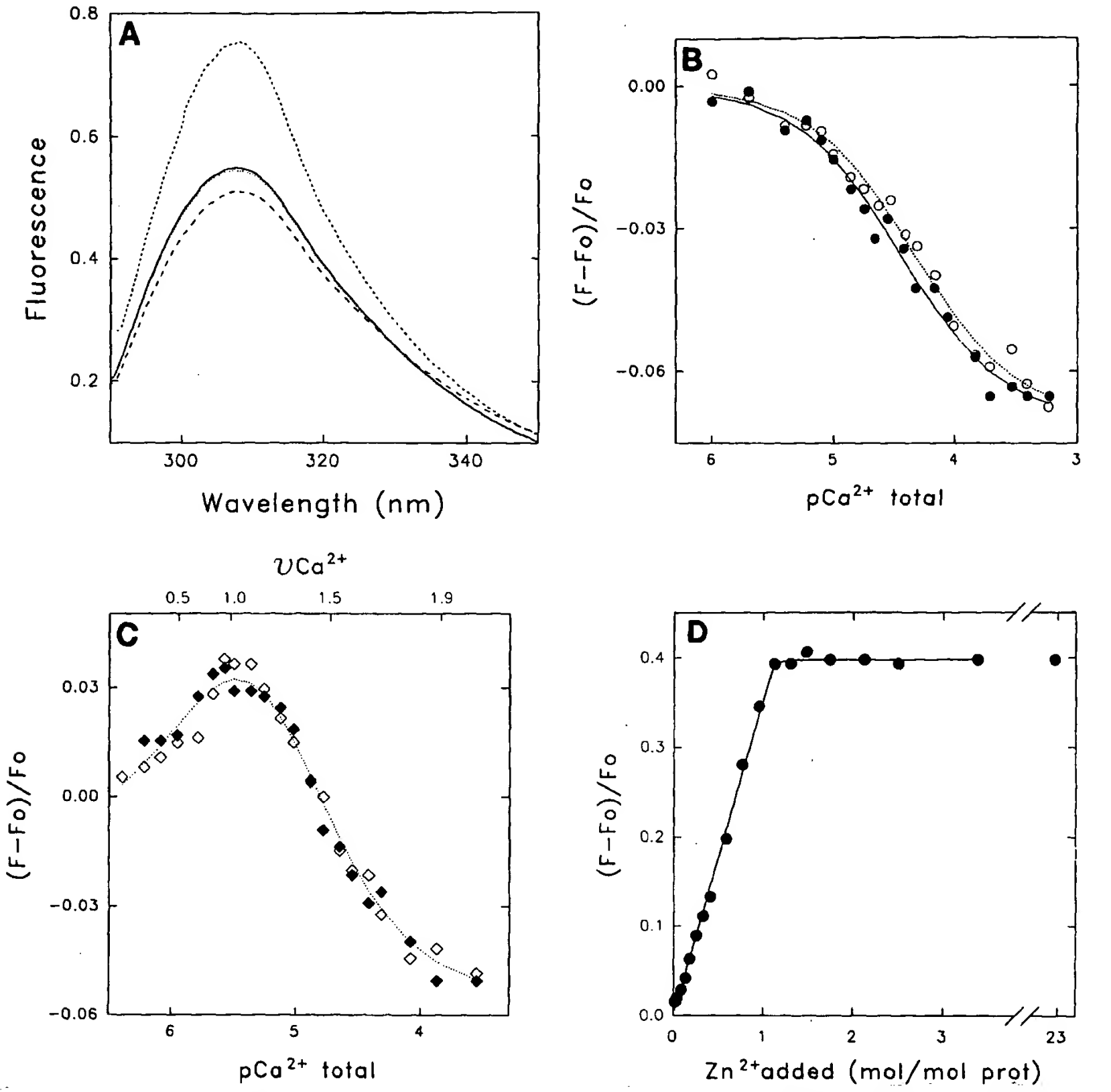

FIG. 6. *A*, fluorescence spectra of calgranulin C (10 μM) in 25 mM Tris-HCl (pH 7.4) with either 2 mM EDTA (*solid line*), 5 mM $MgCl_2$ (*dotted line*), 2 mM $CaCl_2$ (*long-dashed line*), or 0.1 mM $ZnCl_2$ (*short-dashed line*). *B–D*, tyrosine fluorescence titration experiments. Protein concentration was 2.2 μM. In each experiment, values of fluorescence intensity at 308 nm (*F*) were normalized to those corresponding to zero ligand concentration (F_o). *B*, titration of apo-calgranulin C with Ca^{2+} in the absence (*closed circles*) or in the presence (*open circles*) of 5 mM $MgCl_2$. The curves were calculated by fitting Equation 3 to the experimental points. *C*, titration of Zn^{2+}-bound calgranulin C with Ca^{2+} in the absence (*closed diamonds*) or in the presence (*open diamonds*) of 5 mM $MgCl_2$. The top of the panel indicates the corresponding amount of Ca^{2+} bound per mol of calgranulin C monomer (νCa^{2+}) as calculated from the binding data of Fig. 5. *D*, titration of apo-calgranulin C with Zn^{2+}.

and could be eluted from the column with 1 mM EDTA (data not shown).

DISCUSSION

In this paper we report the characterization of calgranulin C, a new member of the S100 protein family. Members of this family are acidic CaBPs about 100 residues in length. They contain two EF-hand motifs per monomer, the first having an unusual 14-residue calcium-binding loop that is distinctive of this family (2, 38). Most S100 proteins are expressed in a tissue-specific and cell cycle-specific fashion, this leading to the proposal that they are involved in cell differentiation and cell cycle progression (4, 39–41). Moreover, some S100 proteins such as CACY and CAPL are associated with tumor development and the induction of metastasis (42, 43). Other functions postulated for S100 proteins include the regulation of cytosolic Ca^{2+} concentration, inhibition of specific phosphorylation events, and modulation of cytoskeletal-membrane interactions (4, 44, 45).

Calgranulin C was purified from pig granulocytes by a simple procedure involving gel filtration and anion exchange chromatography. As judged from the amount of pure protein obtained by this method, calgranulin C comprises at least 8% of pig granulocyte cytosolic proteins. This percentage could be even higher since losses inherent in the chromatographic steps were not taken into account. The protein was also purified from lymphocyte extracts,

although the amount of pure calgranulin C obtained per mg of total protein was 50–60 times smaller than that obtained from granulocytes. In fact, this low content of calgranulin C in the lymphocyte extracts can be explained by the presence of contaminating granulocytes (usually 1–3%) in the lymphocyte preparations used. Therefore, there is a possibility that pig lymphocytes may not express calgranulin C at all.

Fig. 8*A* shows the multiple sequence alignment of the S100 protein family. The amino acid identity between calgranulin C and the other S100 proteins ranges from 27% (S100E) to 45% (calgranulin B). The least conserved segments are the C-terminal region and the "hinge" region that connects the two EF-hands (Fig. 8*A*). These regions are thought to provide specificity to the function of each S100 protein (4). The phylogenetic tree of the S100 family is shown in Fig. 8*B*. Calgranulin C appears to be most closely related to calgranulin B, a protein that is also expressed in granulocytes. However, calgranulin B forms tightly associated heterocomplexes with calgranulin A (23–25) whereas no such heterocomplex formation is observed for calgranulin C. Furthermore, the unusually long C-terminal "tail" characteristic of calgranulin B is absent in calgranulin C (Fig. 8*A*). It is worth mentioning that this "tail" is phosphorylated upon neutrophil activation (24, 46) and that such a phosphorylation event is thought to be important for the function of calgranulin B (41). These structural differences between calgranulins B and C suggest that these proteins may have separate functions in granulocytes.

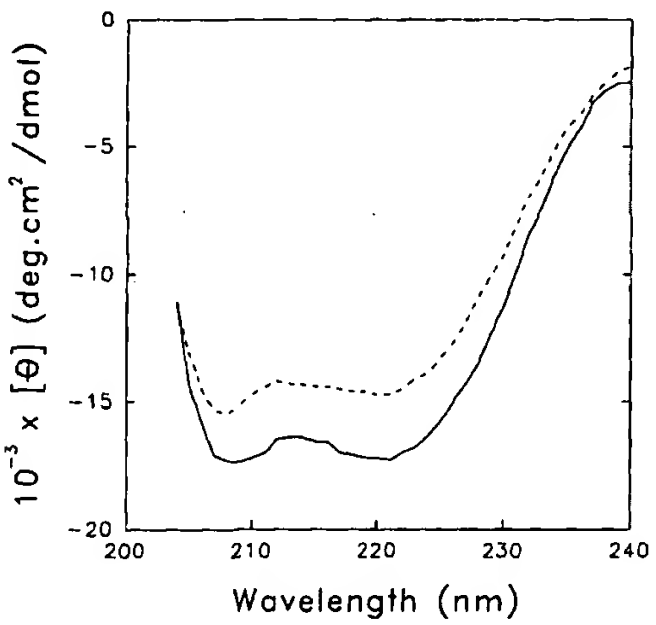

FIG. 7. **Far-UV circular dichroism of calgranulin C.** Spectra were obtained in 50 mM Tris-HCl (pH 7.4) in the presence of either 2 mM EDTA (*solid line*) or 2 mM $CaCl_2$ (*dashed line*). Protein concentration was 50 μM.

Although purified calgranulin C appeared homogeneous by SDS-PAGE and N-terminal sequencing, its ESMS spectrum showed the presence of two components. The molecular mass of the major component (10,614 Da) fits the value calculated from the primary structure, while the minor one is approximately 40 Da heavier. As many EF-hand CaBPs retain their ligand during purification (47), it is likely that this difference in mass is due to a single Ca^{2+} bound to the protein. Isoelectric focusing analysis of calgranulin C also shows the presence of two components, although in this case analyses performed in the presence of Ca^{2+}, EDTA and denaturing agents strongly suggest that the charge heterogeneity is not a consequence of ligand binding. Further experiments will be required to elucidate this point.

Most S100 proteins are known to exist as dimers, and both disulfide-bound (35, 48) and noncovalently associated forms (9, 23) have already been described. Cross-linking experiments and gel filtration analysis of purified calgranulin C demon-

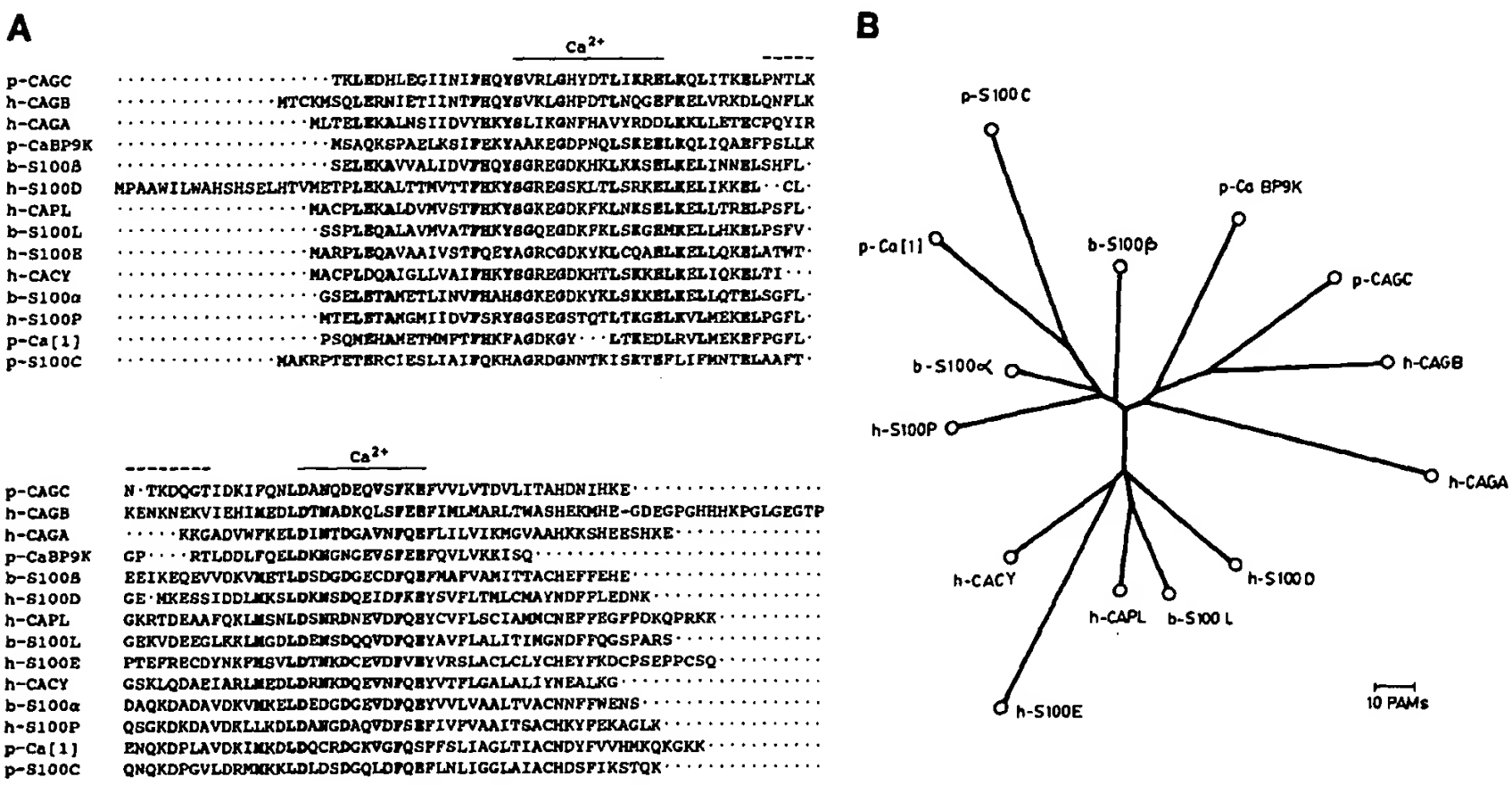

FIG. 8. **Sequence analysis of the S100 protein family.** Amino acid sequences were compared by using the Darwin system (32). The following sequences were obtained from the SWISS-PROT protein data bank (accession codes given in parentheses): *h-CAGB*, human calgranulin B (P06702); *h-CAGA*, human calgranulin A (P05109); *p-CaBP9K*, pig calbindin-D9K (P02632); *b-S100β*, bovine S100 protein β-chain (P02638); *h-CAPL*, human placental calcium-binding protein (P26447); *b-S100L*, bovine S100L (P10462); *h-CACY*, human calcyclin (P06703); *b-S100α*, bovine S100 protein α-chain (P02639); *h-S100P*, human S100P (P25815); *p-Ca[1]*, pig calpactin I light chain (P04163). The other sequences are: *p-CAGC*, pig calgranulin C (this work); *h-S100D*, human S100D (38); *h-S100E*, human S100E (38); *p-S100C*, pig S100C (54). *A*, multiple sequence alignment. The calcium-binding loops of the two EF-hand motifs and the segment connecting both motifs are indicated with *solid* and *dashed lines*, respectively. Residues common to at least 10 sequences are shown in *bold* characters. *B*, phylogenetic tree. The branch lengths are in PAMs (accepted point mutations per 100 residues).

strate the presence of a homodimeric form apart from the monomer. The fact that the amino acid sequence of calgranulin C contains no cysteine residues indicates that the homodimer is noncovalently associated. Whether the dimeric or monomeric forms occur *in vivo* remains to be established.

The binding properties of calgranulin C were studied by means of a direct $^{45}Ca^{2+}$-binding assay and by tyrosine fluorescence titration. Our results indicate that both Ca^{2+} and Zn^{2+} are bound to calgranulin C and that the binding of Zn^{2+} induces a profound change in the Ca^{2+}-binding properties of the protein. In the absence of zinc, the protein appears to bind 1 Ca^{2+}/monomer with a K_a of approximately 2×10^4 M^{-1}. In contrast, Zn^{2+}-loaded calgranulin C binds 2 Ca^{2+}/monomer with stoichiometric binding constants in the order of 10^7 and 10^4 M^{-1}, respectively. The binding of 2 Ca^{2+}/calgranulin C monomer is consistent with the presence of two EF-hand motifs in the amino acid sequence. Both EF-hands seem to be specific for Ca^{2+} as the fluorescence titration curves were minimally affected by 5 mM Mg^{2+}. As also inferred from tyrosine fluorescence titration, calgranulin C has an additional binding site with high affinity for Zn^{2+}. It is worth noting that the C-terminal region of calgranulin C contains a His-*X*-*X*-*X*-His motif comprising residues 85–89 (Fig. 4). This motif within an α-helix has two correctly positioned imidazoles that can chelate a zinc ion (49). As the secondary structure prediction by means of the PHD algorithm (50) suggests that residues 85–89 are within an α-helix (not shown), we propose that the side chains of both His^{85} and His^{89} may participate in the binding of the zinc ion.

The ability of an S100 protein to bind Zn^{2+} with high affinity and the zinc-induced increase in calcium-binding affinity have been previously described only for S100$\beta\beta$ (33). As proposed for this protein, calgranulin C should be considered both as a calcium- and zinc-binding protein. Whether Ca^{2+} binding to calgranulin C is regulated *in vivo* by Zn^{2+} remains to be determined.

The function of calgranulin C in granulocytes is unknown. In addition to its possible role as a Ca^{2+} buffer due to its high concentration, it may be involved in specific calcium-dependent signal transduction pathways. According to the currently accepted mechanism of action of EF-hand CaBPs in Ca^{2+} signal transduction (4, 5), calgranulin C should undergo Ca^{2+}-dependent conformational changes responsible for the transmission of information to effector proteins. Such conformational changes were reported for some members of the S100 protein family, namely S100$\alpha\beta$, S100$\beta\beta$, and S100P (9, 12, 47). In contrast, calbindin-D9K, which has been suggested to act merely as a Ca^{2+} buffer (51), undergoes only very subtle conformational changes upon calcium binding (52). These considerations prompted us to investigate whether the conformation of calgranulin C is affected by calcium. Our results indicate a Ca^{2+}-induced change in the environment of at least 1 of the 2 tyrosine residues of the molecule. Moreover, binding of Ca^{2+} to the protein causes a significant decrease in the apparent α-helix content, which is certainly in line with previous observations on other S100 proteins (9, 11, 47). Finally, the fact that Ca^{2+}-loaded calgranulin C is retained on a phenyl-Superose column and can be eluted with EDTA suggests that the protein exposes a hydrophobic region in the presence of calcium. Taken together, these results demonstrate that calgranulin C undergoes a gross conformational change upon calcium binding and support the possibility that this novel protein is involved in Ca^{2+}-dependent signal transduction events.

Intracellular calcium levels modulate many phagocyte functions including chemotaxis, phagocytosis, degranulation, and the generation of reactive oxygen species (13–16). In addition to calgranulin C, various putative mediators of the calcium signal in granulocytes have been identified, namely calmodulin (19), a 33-kDa annexin protein (17), grancalcin (18), and the calgranulin A/B heterocomplex (53). The notion that each protein may transmit the calcium signal to a different cellular effector deserves future investigation. Ongoing studies are aimed at identifying the cellular target of calgranulin C.

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REFERENCES

1. Carafoli, E. (1987) *Annu. Rev. Biochem.* **56,** 395–433
2. Heizmann, C. W., and Hunziker, W. (1991) *Trends Biochem. Sci.* **13,** 98–103
3. Burgoyne, R. D., and Morgan, A. (1992) *Biochem. Soc. Trans.* **20,** 834–836
4. Kligman, D., and Hilt, D. C. (1988) *Trends Biochem. Sci.* **13,** 437–443
5. da Silva, A. C. R., and Reinach, F. C. (1991) *Trends Biochem. Sci.* **16,** 53–57
6. Hinrichsen, R. D. (1993) *Biochim. Biophys. Acta* **1155,** 277–293
7. LaPorte, D., Wierman, B., and Storm, D. (1980) *Biochemistry* **19,** 3814–3819
8. Tanaka, T., and Hidaka, H. (1980) *J. Biol. Chem.* **255,** 11078–11080
9. Baudier, J., and Gerard, D. (1986) *J. Biol. Chem.* **261,** 8204–8212
10. Strynadka, N. C. J., and James, M. N. G. (1989) *Annu. Rev. Biochem.* **58,** 951–998
11. Mani, R. S., and Kay, C. M. (1990) *Biochemistry* **29,** 1398–1404
12. Becker, T., Gerke, V., Kube, E., and Weber, K. (1992) *Eur. J. Biochem.* **207,** 541–547
13. Snyderman R., and Goetzl, E. J. (1981) *Science* **213,** 830–837
14. Smolen, J. E., Korchak, H. M., and Weissmann, G. (1981) *Biochim. Biophys. Acta* **677,** 512–520
15. Lew, P. D., Wollheim, C. B., Waldvogel, F. A., and Pozzan, T. (1984) *J. Cell Biol.* **99,** 1212–1220
16. Sawyer, D. W., Sullivan, J. A., and Mandell, G. L. (1985) *Science* **230,** 663–666
17. Ernst, J. D., Hoye, E., Blackwood, R. A., and Jaye, D. (1990) *J. Clin. Invest.* **85,** 1065–1071
18. Boyhan, A., Casimir, C. M., French, J. K., Teahan, C. G., and Segal, A. W. (1992) *J. Biol. Chem.* **267,** 2928–2933
19. Jones, H. P., Ghai, G., Petrone, W. F., and McCord, J. (1982) *Biochim. Biophys. Acta* **714,** 152–156
20. Odink, K., Cerletti, N., Brüggen, J., Clerc, R. G., Tarcsay, L., Zwadlo, G., Gerhards, G., Schlegel, R., and Sorg, C. (1987) *Nature* **330,** 80–82
21. Wilkinson, M. M., Busuttil, A., Hayward, C., Brock, D. J. H., Dorin, J. R., and van Heyningen, V. (1988) *J. Cell Sci.* **91,** 221–230
22. Schleicher, C. H., Dell'Angelica, E. C., and Santomé, J. A. (1993) *Int. J. Biochem.* **25,** 1251–1256
23. Teigelkamp, S., Bhardwaj, R. S., Roth, J., Meinardus-Hager, G., Karas, M., and Sorg, C. (1991) *J. Biol. Chem.* **266,** 13462–13467
24. Dianoux, A.-C., Stasia, M.-J., Garin, J., Gagnon, J., and Vignais, P. V. (1992) *Biochemistry* **31,** 5898–5905
25. Schleicher, C. H., Dell'Angelica, E. C., and Santomé, J. A. (1994) *FASEB J.* **8,** A1351
26. Bruns, G. A. P., and Sherman, S. L. (1989) *Cytogenet. Cell Genet.* **51,** 67–90
27. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
28. Chakrabartty, A., Kortemme, T., Padmanabhan, S., and Baldwin, R. L. (1993) *Biochemistry* **32,** 5560–5565
29. Brandts, J. F., and Kaplan, L. J. (1973) *Biochemistry* **12,** 2011–2024
30. Schägger, H., and von Jagow, G. (1987) *Anal. Biochem.* **166,** 368–379
31. Admon, A., and King, D. S. (1992) in *Techniques in Protein Chemistry III* (Angeletti, R. H., ed) pp. 45–51, Academic Press, San Diego
32. Gonnet, G. H. (1992) *A Tutorial Introduction to Computational Biochemistry Using Darwin*, Swiss Federal Institute of Technology (ETH), Zurich
33. Baudier, J., Glasser, N., and Gerard, D. (1986) *J. Biol. Chem.* **261,** 8192–8203
34. Zhong, L., and Johnson, C. W., Jr. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 4462–4465
35. Pedrocchi, M., Schäfer, B. W., Durussel, I., Cox, J. A., and Heizmann, C. W. (1994) *Biochemistry* **33,** 6732–6738
36. Thornton, J. M., and Taylor, W. R. (1989) in *Protein Sequencing: A Practical Approach* (Findlay, J. B. C., and Geisow, M. J., eds) pp. 147–190, IRL Press, Oxford
37. Kyte, J., and Doolittle, R. F. (1982) *J. Mol. Biol.* **157,** 105–132
38. Engelkamp, D., Schäfer, B. W., Mattei, M. G., Erne, P., and Heizmann, C. W. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 6547–6551
39. Calabretta, B., Kaczmarek, L., Mars, W., Ochoa, D., Gibson, C. W., Hirschhorn, R. R., and Baserga, R. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 4463–4467
40. Lagasse, E., and Weissman, I. L. (1992) *Blood* **79,** 1907–1915
41. Hessian, P. A., Edgeworth, J., and Hogg, N. (1993) *J. Leukocyte Biol.* **53,** 197–204
42. Calabretta, B., Venturelli, D., Kaczmarek, L., Narni, F., Talpaz, M., Anderson, B., Beran, M., and Baserga, R. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 1495–1498
43. Davies, B. R., Davies, M. P. A., Gibbs, F. E. M., Barraclough, R., and Rudland, P. S. (1993) *Oncogene* **8,** 999–1008
44. Murao, S., Collart, F. R., and Huberman, E. (1989) *J. Biol. Chem.* **264,** 8356–8360

45. Roth, J., Burwinkel, F., van den Bos, C., Goebeler, M., Vollmer, E., and Sorg, C. (1993) *Blood* **82**, 1875–1883
46. Edgeworth, J., Freemont, P., and Hogg, N. (1989) *Nature* **342**, 189–192
47. Mani, R. S., and Kay, C. M. (1987) *Methods Enzymol.* **139**, 168–187
48. Kligman, D., and Marshak, D. R. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 7136–7139
49. Higaki, J. N., Fletterick, R. J., and Craik, C. S. (1992) *Trends Biochem. Sci.* **17**, 100–104
50. Rost, B., and Sander, C. (1993) *J. Mol. Biol.* **232**, 584–599
51. Christakos, S., Gabrielides, C., and Rhoten, W. B. (1989) *Endocr. Rev.* **10**, 3–26
52. Skelton, N. J., Kördel, J., Akke, M., Forsén, S., and Chazin, W. J. (1994) *Nature Struct. Biol.* **1**, 239–245
53. Edgeworth, J., Gorman, M., Bennett, R., Freemont, P., and Hogg, N. (1991) *J. Biol. Chem.* **266**, 7706–7713
54. Ohta, H., Sasaki, T., Naka, M., Hiraoka, O., Miyamoto, C., Furuichi, Y., and Tanaka, T. (1991) *FEBS Lett.* **295**, 93–96

ACCESSION NUMBER: 1994-0071153 PASCAL

TITLE (IN ENGLISH): A monoclonal antibody to calcitonin gene-related peptide abolishes capsaicin-induced gastroprotection
AUTHOR: PESKAR B. M.; WONG H. C.; WALSH J. H.; HOLZER P.
CORPORATE SOURCE: Ruhr-univ. Bochum, dep. exp. clin. medicine, 44780 Bochum, Germany, Federal Republic of
SOURCE: European journal of pharmacology, (1993), 250(1), 201-203, 10 refs.
ISSN: 0014-2999 CODEN: EJPHAZ
DOCUMENT TYPE: Journal; Short communication
BIBLIOGRAPHIC LEVEL: Analytic
COUNTRY: Netherlands
LANGUAGE: English
AVAILABILITY: INIST-13322, 354000023763260330
AB Calcitonin gene-related **peptide** (CGRP) released from vasodilator nerves is implicated in the gastroprotective action of capsaicin. This experimental paradigm was used to prove the effectiveness of a monoclonal **anti-CGRP antibody**. The experiments were performed in anaesthetized rats in which intragastric capsaicin (0.5 mg /kg) reduced gastric injury due to ethanol (50%) by 72%. The protective effect of capsaicin was abolished by close arterial administration of the **anti-CGRP antibody** #4901 (5 mg) to the stomach. A monoclonal antibody to keyhole limpet haemocyanin was without effect. These data establish **anti-CGRP antibody** #4901 as a tool to neutralize endogenously released CGRP and show that CGRP is indispensable for the gastroprotective action of capsaicin
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L13 ANSWER 72 OF 117 SCISEARCH COPYRIGHT (c) 2005 The Thomson Corporation. on STN

ACCESSION NUMBER: 94:432044 SCISEARCH

THE GENUINE ARTICLE: NV610

TITLE: CALCITONIN-GENE-RELATED **PEPTIDE** (CGRP) AS AN ENDOGENOUS VASODILATOR - IN-VIVO IMMUNOBLOCKADES STUDIES WITH AN **ANTI-CGRP** MONOCLONAL-ANTIBODY AND ITS FAB' **FRAGMENT**

AUTHOR: TAN K K C (Reprint); BROWN M J; HARGREAVES R J; SHEPHEARD S L; COOK D A; HILL R G

CORPORATE SOURCE: ADDENBROOKES HOSP, CLIN PHARMACOL UNIT, CAMBRIDGE CB2 2QQ, ENGLAND; MERCK SHARP & DOHME LTD, NEUROSCI RES CTR, HARLOW CM20 2QR, ESSEX, ENGLAND

COUNTRY OF AUTHOR: ENGLAND

SOURCE: BRITISH JOURNAL OF PHARMACOLOGY, (JUL 1994) Vol. 112, Supp. S, pp. U14.
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TI CALCITONIN-GENE-RELATED **PEPTIDE** (CGRP) AS AN ENDOGENOUS VASODILATOR - IN-VIVO IMMUNOBLOCKADES STUDIES WITH AN **ANTI-CGRP** MONOCLONAL-ANTIBODY AND ITS FAB' **FRAGMENT**

ANSWER 67 OF 117 USPATFULL on STN

ACCESSION NUMBER: 94:26633 USPATFULL

TITLE: Antibodies to islet amyloid polypeptide (IAPP) and subunits thereof

INVENTOR(S): Westermark, Per, Balinge, Sweden
Johnson, Kenneth H., Minnespolis, MN, United States

PATENT ASSIGNEE(S): Regents of the University of Minnesota, Minneapolis, MN, United States (U.S. corporation) a part interest

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 5298605		19940329
APPLICATION INFO.:	US 1992-838043		19920219 (7)

RELATED APPLN. INFO.: Division of Ser. No. US 1991-658442, filed on 21 Feb 1991, now patented, Pat. No. US 5112945 which is a continuation of Ser. No. US 1987-105267, filed on 7 Oct 1987, now abandoned

	NUMBER	DATE
PRIORITY INFORMATION:	SE 1986-4270	19861008

DOCUMENT TYPE: Utility

FILE SEGMENT: Granted

PRIMARY EXAMINER: Nucker, Christine M.

ASSISTANT EXAMINER: Cunningham, T.

LEGAL REPRESENTATIVE: Merchant, Gould, Smith, Edell, Welter & Schmidt

NUMBER OF CLAIMS: 11

EXEMPLARY CLAIM: 1

NUMBER OF DRAWINGS: 1 Drawing Figure(s); 1 Drawing Page(s)

LINE COUNT: 614

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB This invention is directed to antibodies which react with human islet amyloid polypeptide and which do not significantly react with insulin or calcitonin gene-related peptides. Preparations of antibodies are provided which bind to islet amyloid polypeptide (IAPP) which is substantially free of islet amyloid, and when isolated from humans, has the following amino acid sequence in positions 1-37:

Lys-Cys-Asn-Thr-Ala-Thr-Cys-Ala-Thr-Gln-Arg-Leu-Ala-Asn-Phe-Leu-Val-His-Ser-Ser-Asn-Asn-Phe-Gly-Ala-Ile-Leu-Ser-Ser-Thr-Asn-Val-Gly-Ser-Asn-Thr-Tyr.

SUMM . . . of the invention has no significant reaction with e.g. insulin (native form, A- or B-chain thereof) and calcitonin gene related **peptides** (**CGRPs**). **Anti**-IAPP antibodies may be produced in a manner as is common practice for antibodies, by means of immunizing a suitable animal. . . etc.) with an IAPP-immunogen, followed by working up the resultant antibodies to obtain a desired purity and form thereof (derivatives, **fragments** etc.). The production of the anti-IAPP antibodies may be accomplished by known monoclonal technique. The anti-IAPP antibodies of the antibody. . .

ANSWER 61 OF 117 USPATFULL on STN

ACCESSION NUMBER:	95:52267 USPATFULL
TITLE:	Kit for detection of islet amyloid polypeptide (IAPP)
INVENTOR(S):	Westermark, Per, Balinge, Sweden Johnson, Kenneth H., Minneapolis, MN, United States
PATENT ASSIGNEE(S):	Regents of the University of Minnesota, Minneapolis, MN, United States (U.S. corporation)

	NUMBER	KIND	DATE	
PATENT INFORMATION:	US 5424221		19950613	
APPLICATION INFO.:	US 1994-183150		19940118	(8)
DISCLAIMER DATE:	20200329			
RELATED APPLN. INFO.:	Division of Ser. No. US 1992-838043, filed on 19 Feb 1992, now patented, Pat. No. US 5298605 which is a division of Ser. No. US 1991-658442, filed on 21 Feb 1991, now patented, Pat. No. US 5112945 which is a continuation of Ser. No. US 1987-105267, filed on 7 Oct 1987, now abandoned			

	NUMBER	DATE
PRIORITY INFORMATION:	SE 1986-4270	19861008

DOCUMENT TYPE:	Utility
FILE SEGMENT:	Granted
PRIMARY EXAMINER:	Saunders, David
ASSISTANT EXAMINER:	Wilson, Eve J.
LEGAL REPRESENTATIVE:	Schwegman, Lundberg & Woessner
NUMBER OF CLAIMS:	13
EXEMPLARY CLAIM:	1
NUMBER OF DRAWINGS:	1 Drawing Figure(s); 1 Drawing Page(s)
LINE COUNT:	669

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB This invention is directed to kits for the detection of human islet amyloid polypeptide (IAPP) comprising (a) purified preparations of antibodies which react specifically with insulin or calcitonin gene-related peptides and (b) a preselected amount of human islet amyloid polypeptide which is essentially free of islet amyloid, which polypeptide is one subunit of islet amyloid and which is prepared by depolymerizing human islet amyloid; or a preselected amount of human islet amyloid polypeptide which is essentially free of islet amyloid and has the amino acid sequence: lys-cys-Asn-Thr-Ala-Thr-Cys-Ala-Thr-Gln-Arg-Leu-Ala-Asn-Phe-Leu-Val-His-Ser-Ser-Asn-Asn-Phe-Gly-Ala-Ile-Leu-Ser-Ser-Thr-Asn-Val-Gly-Ser-Asn-Thr-Tyr.

SUMM . . . of the invention has no significant reaction with e.g. insulin (native form, A- or B-chain thereof) and calcitonin gene related **peptides** (**CGRPs**). **Anti**-IAPP antibodies may be produced in a manner as is common practice for antibodies, by means of immunizing a suitable animal. . . etc.) with an IAPP-immunogen, followed by working up the resultant antibodies to obtain a desired purity and form thereof (derivatives, **fragments** etc.). The production of the anti-IAPP antibodies may be accomplished by known monoclonal techniques. The anti-IAPP antibodies of the antibody. . .